

MECHANISMS OF HYDROGEN

PEROXIDE FORMATION BY

LEUKOCYTES

CENTRE FOR NEWFOUNDLAND STUDIES

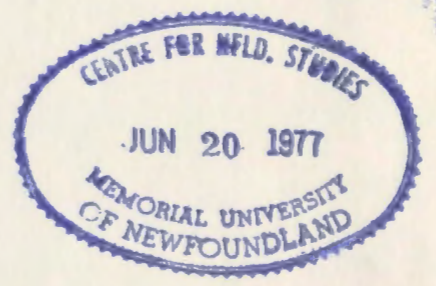
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MECHANISMS OF HYDROGEN PEROXIDE FORMATION
BY LEUKOCYTES

A thesis submitted

by



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requirements for
the degree of

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-Les gens ont des étoiles qui ne sont pas les mêmes. Pour les uns, qui voyagent, les étoiles sont des guides. Pour d' autres elles ne sont rien que de petites lumières. Pour d' autres qui sont savants elles sont des problèmes. Pour mon businessman elles étaient de l' or. Mais toutes ces étoiles-là se taisent.

Toi, tu auras des étoiles comme personne n' en a

"Le Petit Prince" par Antoine de Saint-Exupéry

-All men have the stars, but they are not the same things for different people. For some, who are travelers, the stars are guides. For others they are no more than little lights in the sky. For others, who are scholars, they are problems. For my businessman they were wealth. But all these stars are silent. Y

You - you alone - will have the stars as no one else has them

Antoine de Saint-Exupéry from "The Little Prince"

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ABBREVIATIONS

NADH	: reduced nicotinamide adenine dinucleotide
NADPH	: reduced nicotinamide adenine dinucleotide phosphate
p-CMB	: para-chloromercuribenzoate
HMP	: hexose monophosphate
CGD	: chronic granulomatous disease
2,4-DCP	: 2,4-dichlorophenol
TX-100	: Triton X-100
HRP	: horseradish peroxidase
MPO	: myeloperoxidase
LDH	: lactate dehydrogenase
MDH	: malate dehydrogenase
SOD	: superoxide-dismutase
5'-AMPase	: 5'-nucleotidase
8-ANS	: anilino-8-naphthalene sulfonate
PMN	: polymorphonuclear leukocyte
prot.	: protein
NBT	: Nitroblue tetrazolium
min.	: minute
O.D.	: optical density

ABSTRACT

It is postulated that the increase in H_2O_2 formation following phagocytosis in guinea pig polymorphonuclear leukocytes is due to the activation of a plasma membrane located NAD(P)H oxidase.

The cyanide-resistant oxidase activity of intact leukocytes was markedly stimulated when the leukocytes were suspended in a hypotonic medium. Hydrogen peroxide was the principal product of the oxidase reaction. Furthermore the oxidase was active enough to account for the respiratory burst that accompanies phagocytosis. Evidence that the oxidase activity was located on the outside surface of the plasma membrane was the finding that added NAD(P)H was rapidly oxidized and the plasma membrane was impermeable to NADH or NADPH. Further evidence was the marked inhibition of the oxidase by p-CMB which also did not penetrate the plasma membrane. The oxidase was also inhibited on disruption of the plasma membrane. In addition, the enhanced oxidase activity under hypotonic conditions decreased to normal values when the medium was made isotonic and suggested that a reversible conformational change in the plasma membrane was responsible for the activation of oxidase activities.

Treatment of the leukocytes with digitonin at concentrations low enough so that the leukocytes were not disrupted caused a cyanide-resistant burst of oxygen consumption. Added cytochrome c was reduced and this reduction was blocked by superoxide-dismutase, indicating the formation of superoxide radicals by the leukocytes during the

burst of oxygen consumption.

The cyanide-resistant oxygen consumption of resting PMNs was also found to be stimulated by 2,4-dichlorophenol with H_2O_2 being the sole product formed. NADH and NADPH added to the leukocytes greatly enhanced the oxygen consumption and were oxidized in the process without penetrating the leukocytes. Mn^{2+} stimulated this oxidase activity. Purified peroxidases also had NAD(P)H oxidase activity with similar properties. The NADPH oxidase activity of polymorphonuclear leukocyte granules has not previously been attributed to myeloperoxidase because of its relative insensitivity to cyanide and its activation by aminotriazole. However, the NAD(P)H oxidase activity of myeloperoxidase or horseradish peroxidase was little affected by 2.0 mM cyanide although the peroxidase activity was nearly completely inhibited by 0.1 mM cyanide. Furthermore, the NAD(P)H oxidase activity of myeloperoxidase was considerably enhanced by aminotriazole although the peroxidase activity was inhibited. However, myeloperoxidase is not responsible for the oxidase activity of intact leukocytes as the peroxidase activity of intact leukocytes was very low and density gradient fractionation of the leukocytes showed that the NAD(P)H oxidase activity of the cytosol fraction had very low peroxidase activity.

INTRODUCTION

Phagocytosis is one of the most primitive functions of the cell. Unicellular organisms use the process to ingest nutrients. In multicellular organisms which absorb nutrients through specific organs, phagocytosis has been adapted to play a very important role in biological defence. This phenomenon was pointed out a century ago and studied systematically by Metchnikoff earlier this century (1). In the animal kingdom, the powerful phagocytizing abilities of monocytes and polymorphonuclear leukocytes are employed for biological defence, but the biochemical mechanisms of and metabolic changes induced by phagocytosis have not been clarified as yet.

Several theories about the microbicidal properties of leukocytes are outlined below.

I. Respiration of leukocytes

It is well established that phagocytosis is associated with a burst of oxygen uptake. This phenomenon was first reported in 1933 by Baldrige and Gerard, who observed that the oxygen consumption of leukocytes was increased, and that ingested bacteria were killed in the cells within 15 minutes of incubation (2). The respiratory burst occurs immediately following contact between particles and cells and before extensive phagocytosis has occurred.

The following properties of the respiratory burst have been established:

(i) Inhibitors of mitochondrial respiration, such as KCN and azide do not affect the increased oxygen consumption, although some mitochondrial respiration is inhibited (3, 4). The increased oxygen uptake is, therefore, not due to mitochondrial respiration.

(ii) The respiratory burst starts within a short period (10 - 30 seconds) after the particles make contact with the leukocytes and continues at a constant level for 1 - 30 minutes (5).

(iii) The principal product is hydrogen peroxide (5, 6)

II. Metabolic Bursts

Sbarra and Karnovsky found that the hexose monophosphate shunt (HMP-shunt) is stimulated during phagocytosis (4). In phagocytizing leukocytes the ratio of $^{14}\text{CO}_2$ derived from glucose-1- ^{14}C to that derived from glucose-6- $^{14}\text{CO}_2$ was greatly increased compared with the ratio in resting leukocytes. Two enzymes of the HMP-shunt, glucose-6-phosphate dehydrogenase reduce NADP^+ to NADPH and the maintenance of this pathway is dependent upon the availability of NADP^+ (4, 9). One mechanism for the oxidation of NADPH might be the transfer of electrons by glutathione reductase, glutathione peroxidase and NADPH oxidase (7, 8). Karnovsky et al. (9)

however, have emphasized that glycolysis may be the principal metabolic pathway of carbohydrate metabolism, for even though the HMP-shunt is increased ten fold by phagocytosis; it usually represents an increase in the utilization of carbohydrate by this pathway from 1% to 10% of that metabolized via glycolysis. The rate of synthesis of lipid during phagocytosis (10) and NADPH formed by the HMP-shunt could support the synthesis.

The energy for phagocytosis may come from glycolysis. Phagocytosis is not inhibited by mitochondrial inhibitors such as cyanide, antimycin A or 2,4-dinitrophenol, but is inhibited by glycolytic inhibitors such as NaF or mono-iodoacetic acid (4).

III. Intraleukocytic microbicidal system(s)

The microbicidal action of leukocytes is thought to be due to the hydrogen peroxide formed. The bactericidal activity of leukocytes is depressed by anaerobic conditions, suggesting that the respiratory burst is essential for hydrogen peroxide formation (11). The generation of hydrogen peroxide during phagocytosis was first reported by Iyer, Islam and Quastel in 1961 (3). Subsequently, the formation of hydrogen peroxide by intact leukocytes was confirmed by Paul and Sbarra (6). However, since hydrogen peroxide may be degraded by either intracellular catalase or peroxidase, they were able to detect only a portion of the hydrogen peroxide formed during the phagocytosis process.

Recently Babior et al. demonstrated that superoxide radicals produced by leukocytes during phagocytosis are associated with the increased oxygen uptake and suggested that this may be involved in the bactericidal action (12). The sites of hydrogen peroxide and of superoxide radical formation by leukocytes have not been identified, although there are suggestions that these sites may be in the cytoplasmic granular fractions. If hydrogen peroxide is formed in the cytoplasm it would need to diffuse into the phagosomes; if it is generated in the granules it may be incorporated into the phagocytic vacuole after the granule has fused with the vacuole. However, none of the experimental evidence is sufficient to explain the fact that the respiratory burst occurs within only a few seconds after the particles make contact with the leukocyte membrane.

Once hydrogen peroxide is formed, myeloperoxidase (MPO) plays an important role in the bactericidal reactions in the phagocytic vacuole. Klebanoff and Green have elegantly demonstrated that when radioactive iodide is added to phagocytizing leukocytes, iodine is recovered in a TCA-precipitable fraction, and a water insoluble portion of the fixed iodine is localized in the phagocytic vacuole (13). This iodination reaction is inhibited by peroxidase inhibitors and is low in leukocytes deficient in either MPO or in hydrogen peroxide. It has been suggested that this iodination process is due to myeloperoxidase (14, 15).

Besides the MPO-associated halogenation reaction, there are several other bactericidal factors in leukocytes, such as cationic proteins (16), lipid peroxidation catalysts (17) and lysozyme (18).

IV. Chronic granulomatous disease (CGD)

The first observation of chronic granulomatous disease (CGD) was made by Janeway et al. (1954) in a number of children with increased susceptibility to infectious organisms (19). The condition was reminiscent of γ -globulinemia, but without defective immunoglobulins. Neutrophils from patients with CGD had an impaired ability to kill bacteria and also had decreased virucidal and fungicidal activity. The phagocytosis-associated stimulation of oxygen consumption, glucose-1-¹⁴C oxidation, formate oxidation, neotetrazolium reduction and the iodination reaction were greatly depressed (20, 21, 22).

The importance of the decreased hydrogen peroxide formation by CGD leukocytes is emphasized by the improvement in leukocyte function on the introduction of a hydrogen peroxide generating system into the cells. Micro-organisms which generate hydrogen peroxide are killed by CGD leukocytes (21, 23). Their susceptibility to the intraleukocytic microbicidal system may be due to the replacement of the defective leukocytic hydrogen peroxide generating system with hydrogen peroxide of microbial origin.

V. Possible enzyme system(s) producing H_2O_2 in leukocytes

- (i) Cyanide-resistant NADPH oxidase in granules
- (ii) Cytosol located NADH oxidase
- (iii) Myeloperoxidase

i) For the past decade, Rossi's group have been investigating NADPH oxidase activity in leukocytes and they believe that the cyanide-resistant NADPH oxidase in granules is responsible for the respiratory burst of phagocytosis (25, 24-37). Unlike NADH oxidase, the NADPH oxidase activity is strongly stimulated by phagocytosis. The biochemical properties of the leukocytic NADPH oxidase have been reported as follows:

(a) The K_m of the NADPH oxidase in granules isolated from phagocytizing leukocytes is 0.4 mM, but the K_m of the NADPH oxidase is 4.0 mM when granules are taken from resting leukocytes (29).

(b) The activity of NADPH oxidase is increased over ten fold during phagocytosis (25).

(c) The oxidation of NADPH by isolated granules results in the formation of hydrogen peroxide in the presence of KCN (30).

(d) Surface treatment of leukocytes by phospholipase C or deoxycholate also stimulates the granule-bound NADPH oxidase (28, 32)

(e) Activation of the NADPH oxidase occurs within 30-60 seconds after particles make contact with the surface of leukocytes (37).

From these experimental results, Rossi's group has proposed the following mechanism of hydrogen peroxide formation (37). When particles, such as bacteria, make contact with the leukocytes, a conformational change in the plasma membrane occurs. A signal, transmitted by a chemical mediator from the plasma membrane to the granules, activates NADPH oxidase and results in the respiratory burst of the leukocytes. As a result of NADPH oxidation, the NADP^+ formed stimulates the HMP-shunt. The chemical mediator that transfers the signal and the mechanism of the activation of granule-bound NADPH oxidase has not however been found.

ii) Karnovsky's group, on the other hand, has demonstrated that a cyanide-resistant NADH oxidase exists in the cytosol fraction of leukocytes (38-43). When a homogenate was prepared in 0.34M sucrose, over 50% of the enzyme activity was found in the post-granule fraction. Further evidence favouring NADH oxidase as the key enzyme of hydrogen peroxide generation is that CGD leukocytes have a lower NADH oxidizing activity than normal (23).

The characteristics of NADH oxidase are listed as follows (38):

- (a) pH optimum : 4.5 - 5.0
- (b) substrate specificity : no activity with NADPH
- (c) K_m : 1.0 mM
- (d) Flavin content : 80% of flavin is FAD
- (e) Stoichiometry : $\text{NADH} + \text{H}^+ + \text{O}_2 \rightarrow \text{NAD}^+ + \text{H}_2\text{O}_2$

Karnovsky's group postulated from these observations that NADH oxidase is the principal enzyme associated with

the oxygen burst during the phagocytizing process. They place more emphasis on the Embden-Meyerhof pathway rather than on the HMP-shunt as the source of the hydrogen donor (NADH) for hydrogen peroxide formation. According to them, over 80% of glucose utilization occurs through the Embden-Meyerhof pathway even when the HMP-shunt is increased ten fold, since the HMP-shunt is responsible for only 1-2% of glucose utilization in the resting leukocyte (9).

iii) Other oxidase systems suggested

Quastel and his co-workers in 1964 (44, 45) suggested that the intraleukocytic NADH and NADPH oxidase activities might be myeloperoxidase. They showed that a homogenate of leukocytes oxidized both NADH and NADPH, both oxidations were stimulated by Mn^{2+} and NADPH oxidase was higher in extracts of phagocytizing cells. It has been well established that the peroxidase can oxidize NAD(P)H under aerobic conditions and is activated by Mn^{2+} and some phenols (46). It was once believed that the peroxidation reaction of the peroxidase is inhibited by cyanide, so this could not account for a cyanide-insensitive respiratory burst. In the following it will be shown, however, that myeloperoxidase may act as an NAD(P)H oxidase in the presence of cyanide if H_2O_2 is present.

Another possible oxidase is D-amino acid oxidase (47). This enzyme is found in the granule fraction of leukocytes, and its activity is not inhibited by cyanide. However, the enzyme activity is too small to account for the respiratory burst of the phagocytizing leukocytes.

In summary, the biochemical and morphological observations in chronological order are listed below (Fig 1).

[1] When particles make contact with the surface of a leukocyte, conformational changes in the membrane occur. These can be detected by the 1-anilino-8-naphthalene sulfonate (ANS) fluorescence method (37).

[2] Within 5-10 seconds, the respiratory burst is observed (34).

[3] The HMP-shunt is stimulated (48).

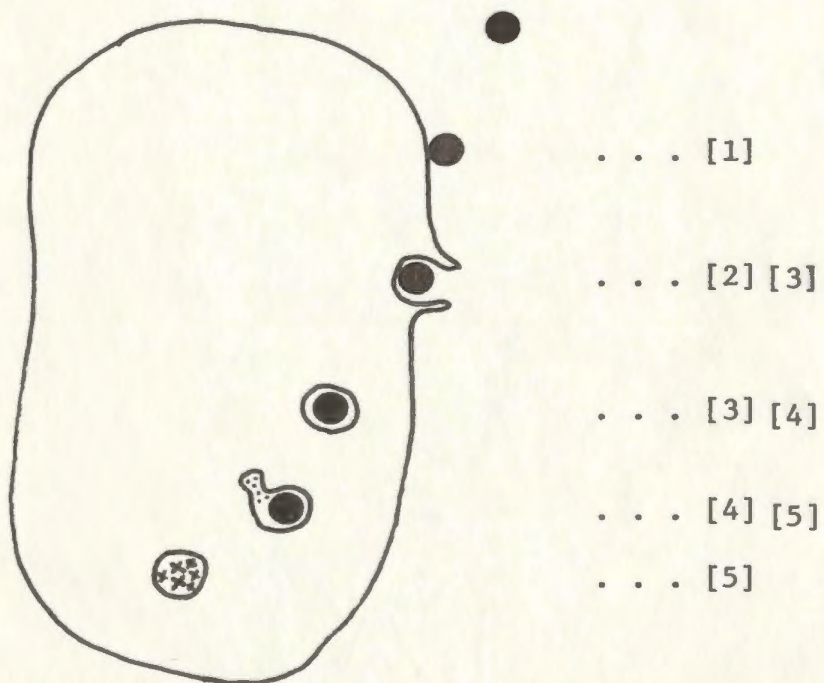
[4] Alkaline phosphatase from secondary granules appears in the phagocytic vacuole (49), and cytochemical studies confirm that secondary granules fuse with the phagosomes within 30-60 seconds of contact with bacteria (49).

[5] Myeloperoxidase reacts with the hydrogen peroxide formed and with a halide as an electron donor so that the bacterial membrane is halogenated. Cytochemical studies confirm that azurophil granules fuse with the phagosomes within 15-30 minutes. Lysosomal acid hydrolases, such as lysozyme, cathepsin and lipase digest the bacteria in these phagolysosomes.

The following questions then arise. What enzyme produces hydrogen peroxide, the principal agent of the microbicidal action of leukocytes; where is it localized and how is it activated?

The aim of the present work was to attempt to answer these questions, for at present, biochemical information

FIGURE 1: Sequence of the phagocytic process



on the hydrogen peroxide producing enzymes and their localization is contradictory. Both NADH oxidase and NADPH oxidase systems have been invoked, and the distribution of both oxidases between cytosol and granules is uncertain.

Chapter 1

MATERIALS AND METHODS

1. Collection of leukocytes

(i) Male guinea pigs were anaesthetized with ether, and were injected intraperitoneally with 30 ml of 0.9% NaCl solution containing 1% neutralized sodium caseinate.

(ii) 14-16 hours later, the animals were again anaesthetized, injected with 30 ml of 0.9% NaCl and the peritoneal fluid was collected, dropwise, through an 18-G hypodermic needle. The yield of leukocytes averaged 3×10^8 cells in a volume of approximately 30 ml. Over 90% of the cells were polymorphonuclear leukocytes. On the average 94% of the cells were polymorphonuclear leukocytes and 6% of them were macrophage. If erythrocytes contaminated the leukocyte preparation, most of them were removed by the following hypotonic shock method (50). Firstly, cells were resuspended in the isotonic solution; two volumes of ice cold water were added and after 30 seconds isotonicity was restored by the addition of 10% NaCl solution.

(iii) The leukocytes were centrifuged at 500 g for 5 minutes at 0-4°C, resuspended and washed with the medium to be employed. When phagocytosis experiments were carried out, Krebs -Ringer phosphate solution was used. For the NAD(P)H oxidase assay 50 mM phosphate buffer (50 mM NaH_2PO_4 - 50 mM K_2HPO_4 , pH 6.0) containing 0.9% NaCl (total osmolarity 0.413 M) was employed. When subfractionation of leukocytes was performed, the washing was carried out with 0.25 M sucrose con-

taining 5 mM sodium bicarbonate (pH 8.0). Further details are provided in the appropriate chapters.

2. Measurement of oxygen consumption

Oxygen uptake was measured with a Clark oxygen electrode in a 1.5 ml vessel at 37°C. The incubation medium for phagocytosis contained the Krebs- Ringer phosphate solution, 2 mM KCN, 1-1.3 mg leukocyte protein and about 10^9 phagocytable particles (bacteria or paraffin oil emulsion). The respiratory burst of leukocytes due to the addition of digitonin or 2,4-dichlorophenol was measured in a similar way except that the particles were omitted and either digitonin or 2,4-DCP was added. When KCN was added, 20 μ l of 150 mM KCN (at pH 7.5) were added to give a final concentration of 2 mM. The respiration of leukocytes in a hypotonic solution was measured with a medium containing 50 mM phosphate buffer (pH 6.0), 0.05 mM MnCl_2 and varying concentrations of NaCl from 0.09% to 0.9% depending upon experimental conditions. The osmolarity varied from 0.136 M to 0.413 M.

3. The NADH and NADPH oxidase assay

NAD(P)H oxidase activity was measured under various conditions by recording the rate of oxygen consumption with a Clark oxygen electrode. The reaction mixture contained 2.0 mM NAD(P)H; 0.05 mM MnCl_2 ; 2 mM KCN; 50 mM phosphate buffer and NaCl concentrations from 0.09 to 0.9%. The uptake of oxygen was measured for 5 minutes after the addition of leuko-

cytes. To obtain the kinetic data, different amounts of NADH or NADPH (final concentration from 0.1 mM to 2.0 mM) were added. Leukocytes inactivated by heating at 80°C for 15 minutes were used as a control.

4. The assay of NADH and NADPH oxidase activated by 2,4-DCP

NAD(P)H oxidase was assayed either as the maximum rate of oxygen consumption or by the decrease in absorbance at 340 nm using the Clark oxygen electrode or the spectrophotometer respectively. The assay medium (final volume 1.5 ml) in the complete system contained the following: 50 mM phosphate buffer (pH 6.0); 0.9% NaCl; 0.15 mg leukocyte protein; 0.1 mM NAD(P)H; 0.05 mM MnCl_2 ; 2 mM KCN and 1.0 mM 2,4-DCP (10 μl of a 150 mM 2,4-DCP in 50% ethanol). The reaction was initiated by the addition of 2,4-DCP and was measured for 5-10 minutes at 37°C. In the spectrophotometric method, similar conditions were employed. When disrupted leukocytes were used, the cells were broken by sonication (50 watts for 60 seconds) at 0°C with a Sonifier model W-185D (Branson sonic power company). To measure the enzymic oxidation of NAD(P)H in the presence of hydrogen peroxide, 1 μmole of H_2O_2 was introduced into the assay system and the reaction was carried out aerobically. As a control, heat-inactivated leukocytes were used.

5. The 2,4-dichlorophenol activated NADH peroxidase

Peroxidase activities were assayed by measuring the oxidation of NADH at 340 nm at 37°C under anaerobic cond-

itions. The reaction medium contained 0.1 mM NADH, 0.3 mM H_2O_2 ; 1 mM 2,4-DCP; 0.9% NaCl and 50 mM phosphate buffer (pH 6.0). The medium was flushed with oxygen-free N_2 gas for 60 seconds and the enzyme was tipped into the reaction mixture from the side arm of the anaerobic cuvette. The rates, expressed as a decrease in NADH per minute, were calculated from the maximum rates obtained during the first five minutes after enzyme addition.

6. Measurement of H_2O_2 formation

When it was necessary to measure H_2O_2 , catalase (Sigma: 35×10^3 IU*) was added to the reaction medium either at the end of the reaction or after terminating the reaction by addition of Triton X-100. Specific details are supplied in the appropriate chapters. Since one cannot measure H_2O_2 in the presence of cyanide by a peroxidase method, the oxygen liberation method was found to be most suitable. The amount of liberated oxygen is a measure of H_2O_2 formed.* (cf. 1 unit decompose $1 \mu\text{mole}$ of H_2O_2 per min at pH 7.0 at 25°C .)

7. Measurement of guaiacol peroxidase activity

Activities of myeloperoxidase were estimated with guaiacol. The assays were carried out in 1.5 ml or 3 ml volume of 50 mM phosphate buffer containing 10 mM guaiacol and 0.3 mM H_2O_2 at 37°C . The increase in absorbance at 470 nm was measured.

8. Latency of dehydrogenases in intact leukocytes

The latency of intracellular dehydrogenases was

measured in the intact leukocytes under different osmotic conditions varying from 0.14 to 0.42 M. Leukocytes were suspended in a medium containing MnCl_2 (0.05 mM), phosphate buffer (50 mM, pH 6.0) and varying concentrations of NaCl (0.09-0.9%). After two minutes incubation, 0.1 ml of the reaction mixture for the oxygen consumption assay system without KCN was placed in a cuvette and lactate dehydrogenase or malate dehydrogenase activities were measured by an adaptation of the methods of Kornberg (51) and Kitts (52), respectively. The reaction mixture for the lactate dehydrogenase assay contained the following: 0.1 ml leukocyte suspension (as described above); 1 mM sodium pyruvate; 0.1 mM NADH; 50 mM phosphate buffer (pH 6.0) and various concentrations of NaCl in a final volume of 1.5 ml. The malate dehydrogenase assay system contained the following: 0.1 ml leukocyte suspension (as described above); 1.0 ml oxaloacetate; 0.1 mM NADH; 50 mM phosphate buffer (pH 6.0) and various concentrations of NaCl in a final volume of 1.5 ml. Activities were determined spectrophotometrically at 340 nm. Total activities of the lactate dehydrogenase and the malate dehydrogenase were estimated using leukocytes disrupted by Triton X-100 (final concentration 0.1%) Similar results were obtained when the cells were disrupted by sonication.

9. Enzyme release

To determine the release of the dehydrogenases from cells, leukocytes treated by various means were centrifuged

at 500 g for 10 minutes, and 0.1 ml samples of the supernatant were assayed.

10. Protein concentration

Protein concentrations were estimated by the method of Lowry (53).

CHAPTER II: NAD(P)H OXIDASE ACTIVITY OF INTACT LEUKOCYTES IN HYPOTONIC MEDIUM

In this chapter, it is postulated that the increase in hydrogen peroxide formation by leukocytes during phagocytosis is due to the activation of a plasma membrane-located NAD(P)H oxidase, since the cyanide-resistant oxidase activity of intact leukocytes is markedly stimulated when leukocytes are suspended in a hypotonic medium.

RESULTS

1. Oxygen consumption and hydrogen peroxide formation

As summarized in Table I, cyanide-resistant oxygen uptake was stimulated when leukocytes were suspended in a hypotonic medium at pH 6.0. Moreover, the addition of 2 mM NADH or NADPH to the hypotonic medium greatly enhanced the oxygen consumption of the leukocytes. Such an increase was not observed in a hypertonic medium.

The cyanide-resistant NAD(P)H oxidase activity and the formation of hydrogen peroxide in the hypotonic medium are shown in Figure 2. When leukocytes were suspended in a hypotonic medium with reduced pyridine nucleotides, the burst of oxygen uptake was initiated after a lag period of 30-60 seconds. The maximum rate was obtained within 1-3 minutes.

Treatment of leukocytes with Triton X-100 (final

concentration 0.1%) caused disruption of the cells, resulting in an inhibition of oxygen consumption. Addition of catalase to the reaction mixture caused the liberation of oxygen, indicating that H_2O_2 was formed in the oxygen-utilization process.

Oxygen, equivalent to 25-35% of the oxygen consumed (when either NADH or NADPH was employed as a hydrogen donor) was liberated by the addition of a large excess of catalase. A simple calculation reveals that 50-70% of the consumed oxygen was converted to H_2O_2 . However, in the absence of KCN, catalase did not liberate oxygen, although nearly the same amount of oxygen was consumed. This lack of accumulation of H_2O_2 in the absence of cyanide may be due to its intraleukocytic breakdown by intracellular catalase. The addition of a large excess of catalase decomposes the hydrogen peroxide even in the presence of cyanide because the inhibition of catalase by cyanide is not instantaneous.

TABLE I

Respiration of PMNs and the activities of NAD(P)H oxidases under different osmotic conditions.

	O_2 UPTAKE ^{a)}	NADPH OXIDASE		NADH OXIDASE	
		O_2 ^{b)}	H_2O_2 ^{c)}	O_2 ^{b)}	H_2O_2 ^{c)}
Isotonic	-KCN	22.1	0.0	0.0	0.0
	+KCN	8.3	0.0	0.0	0.0
Hypotonic	-KCN	26.7	187	143	<6
	+KCN	28.5	213	158	90

TABLE I

Respiration of PMNs and the activities of NAD(P)H oxidases under different osmotic conditions.

- a) Oxygen uptake was measured with 1.3 mg protein of leukocytes in 50 mM phosphate buffer (pH 6.0), 0.9% NaCl (isotonic condition) or 0.09% NaCl (hypotonic condition), and with or without 2 mM KCN. Results are expressed as n-moles oxygen consumed/mg protein/5 minutes.
- b) NAD(P)H oxidase activities represent the rates of further oxygen consumption caused by the addition of reduced pyridine nucleotide. Results are expressed as n-moles oxygen consumed/mg protein/5 minutes. The reaction system contained: 1.3 mg of leukocyte protein, 50 mM phosphate buffer (pH 6.0), 0.05 mM MnCl_2 , 2 mM NAD(P)H, 0.9% NaCl (isotonic conditions) or 0.09% NaCl (hypotonic conditions) and \pm 2 mM KCN. Temperature was 37°C. Reactions were initiated by the addition of leukocytes and followed for 5 minutes.
- c) Results are expressed as n-moles H_2O_2 formed/mg protein/5 minutes.

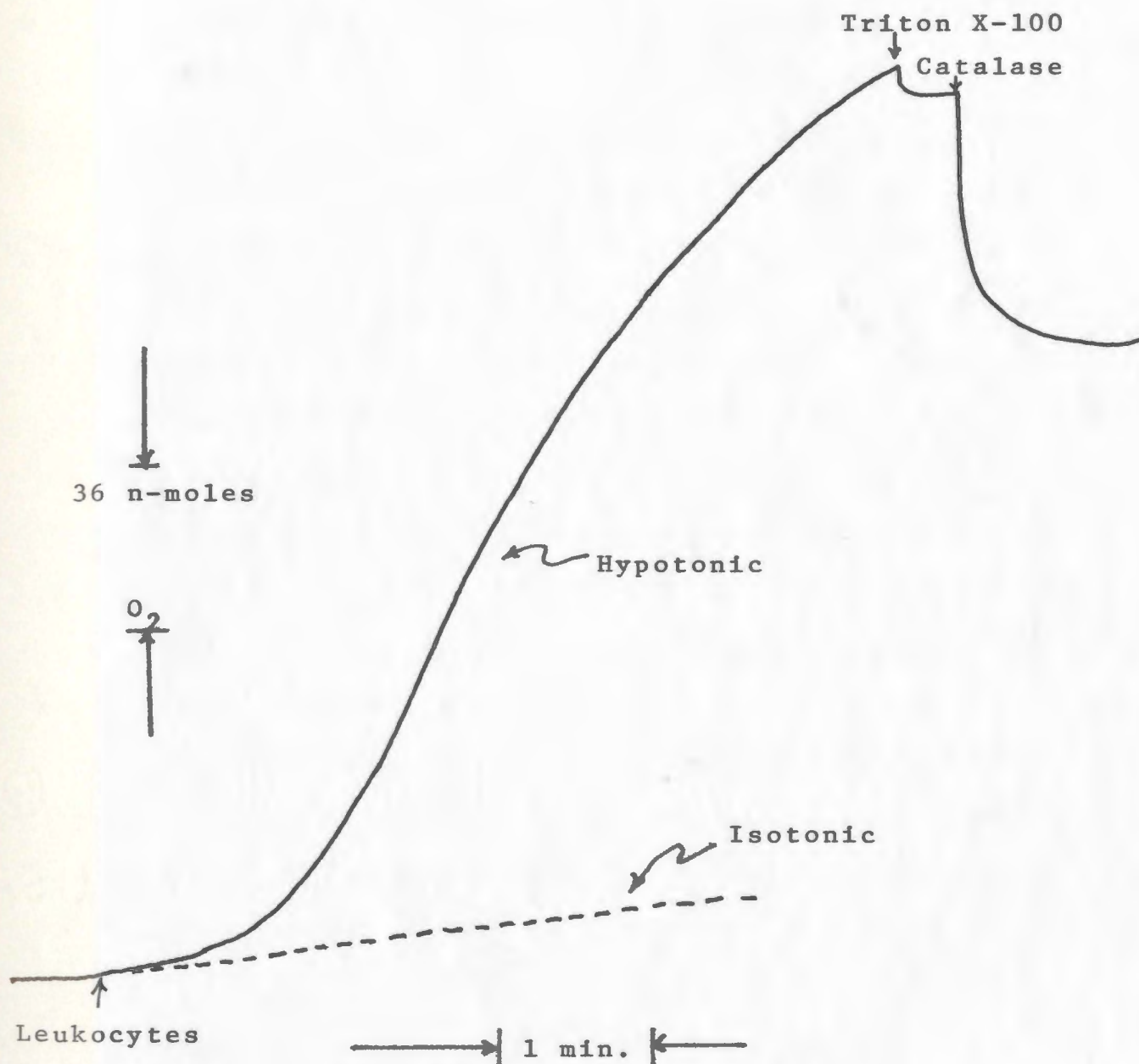


Figure 2

Recording of the oxygen consumption of PMNs in hypotonic medium. Composition of the system: 1.3 mg protein leukocytes, 2 mM KCN, 0.05 mM $MnCl_2$, 2 mM NADPH, 0.09% NaCl, 50 mM phosphate buffer (pH 6.0). After 5 minutes incubation, 0.03 ml of 5% Triton X-100 and catalase were added. Leukocytes in an isotonic medium (0.9% NaCl) were used as a control.

2. The intactness of leukocytes

The results presented in Figure 3 were obtained using the identical assay conditions described in the legend to Figure 2, except that different concentrations of NaCl were employed.

The total amount of oxygen consumption was measured after 5 minutes incubation, and the hydrogen peroxide formed was determined from the oxygen liberated by the addition of catalase at the end of the reaction. The yield of hydrogen peroxide produced by the activated oxidase under hypotonic conditions using either NADH or NADPH as the electron donor was equivalent to about 50-70% of the oxygen consumed. In addition, trypan blue exclusion experiments clearly indicated that over 90% of the leukocytes were intact after 10 minutes in a hypotonic medium.

To determine the permeability of leukocytes to reduced pyridine nucleotides, malate dehydrogenase and lactate dehydrogenase which are localized inside the leukocytes, were measured under different osmotic conditions. There was a modest increase in LDH and MDH activities after hypotonic treatment (Table II). This may indicate that 5-10% of the cells were damaged by this treatment and this agrees with the trypan blue exclusion data (Table III).

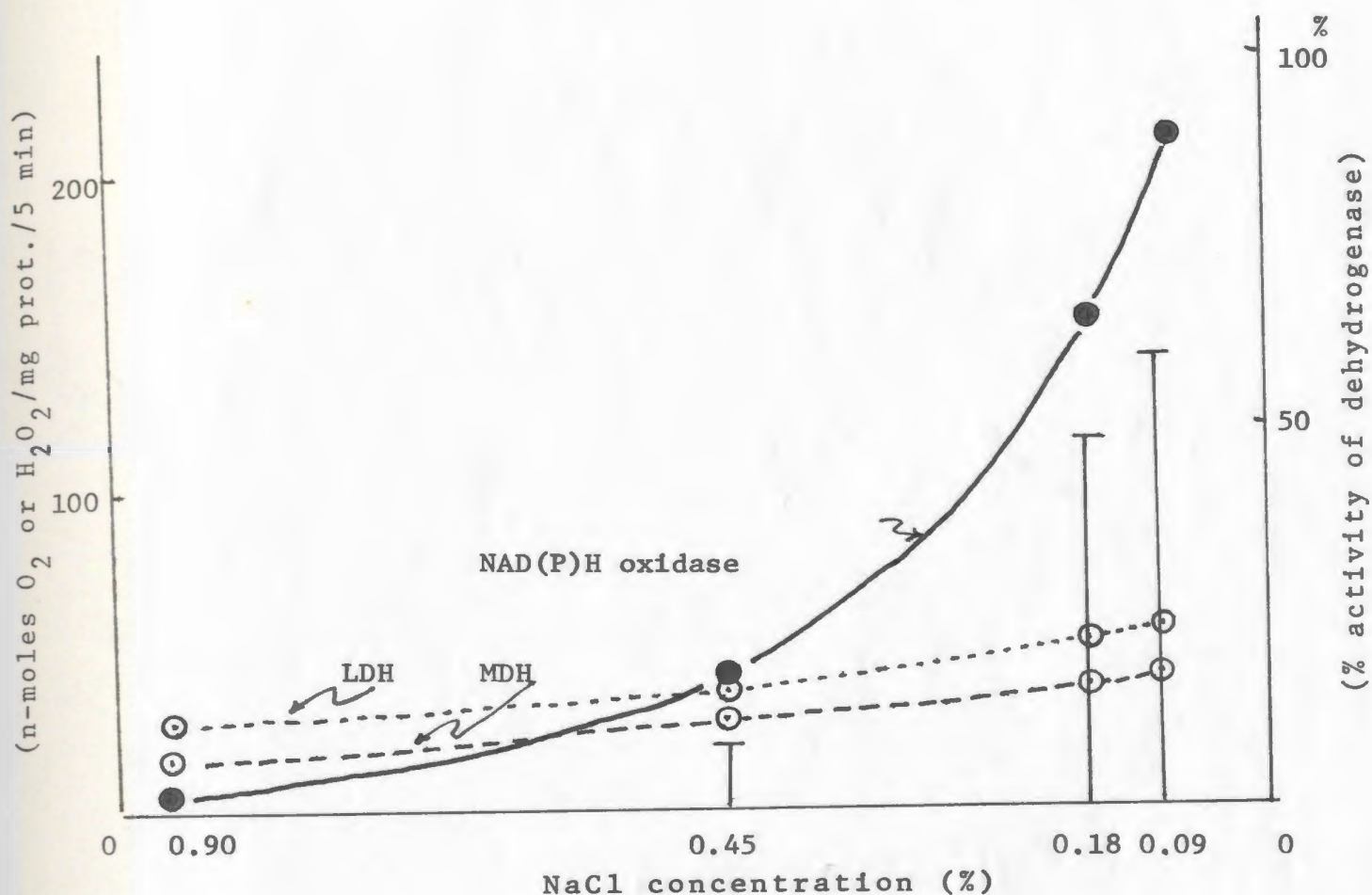


Figure 3

Effects of NaCl concentrations on NAD(P)H oxidases and dehydrogenases.

Activities of lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) were measured by the decrease in absorbance at 340 nm. Medium contained: 0.9-0.09% NaCl, 50 mM phosphate buffer (pH 6.0), 0.05 mM MnCl_2 , 2 mM KCN, 0.1 mM NADH, and 0.1 mg leukocyte protein. Oxygen consumption and H_2O_2 formation were estimated as in Figure 1.

(—●—, NADPH oxidase; --○--, malate dehydrogenase; ---○---, lactate dehydrogenase; T, amount of H_2O_2 formed)

TABLE II

Latency of Intraleukocyte Enzymes

Conditions	Enzyme activity ^{a)} (% of total)		Release of enzymes ^{b)} (% of total)	
	Malate dehydrogenase	Lactate dehydrogenase	Malate dehydrogenase	Lactate dehydrogenase
Isotonic (0.9% NaCl)	6.3	10.5	6.4	5.6
Hypotonic (0.09% NaCl)	15.5	21.0	6.5	6.5
0.1% Triton X-100 (total activity)	100 ^{c)}	100 ^{d)}	100 ^{c)}	100 ^{d)}

a) Leukocytes were suspended in the medium used for NAD(P)H oxidase assay. After 2 minutes incubation at 37°C, enzyme activities were measured.

b) Isotonic, hypotonic and Triton X-100 treated leukocytes were centrifuged at 500 g for 10 minutes and the enzyme activities in supernatant fraction were assayed.

c) Total activity of malate dehydrogenase was 365 n-moles/mg protein/min.

d) Total activity of lactate dehydrogenase was 1430 n-moles/mg protein/min.

TABLE III

Trypan blue exclusion test

Time (minutes)	Isotonic condition % exclusion ^{a)}	Hypotonic condition % exclusion ^{a)}
0	97	96
5	96	93
10	96	91
15	95	88
20	93	90

a) Incubation was carried out in the reaction mixture (1.5 ml) containing 50 mM phosphate buffer (pH 6.0), 0.9% NaCl (isotonic) or 0.09% NaCl (hypotonic) and 1 mg protein of leukocytes at 37°C. After incubation, 0.1 ml of the reaction mixture was added to 0.2 ml of trypan blue (0.1%) solution containing the same concentrations of buffer and salt as the incubation medium. The numbers of stained and non-stained leukocytes were counted after examination with a light microscope.

3. Inhibition of NAD(P)H oxidase by p-chloromercuribenzoate (p-CMB)

As Figure 4 (a) shows, the NAD(P)H oxidase activity of leukocytes in the hypotonic medium was inhibited over 90% by 0.1 mM p-CMB. Lactate dehydrogenase activity in intact cells (isotonic and hypotonic) was not inhibited by an even higher concentration of p-CMB, although it was inhibited when leukocytes were disrupted by treatment with the detergent [Figure 4(b)]. This is consistent with the NAD(P)H oxidase activity being externally located to lactate dehydrogenase provided that these differences in p-CMB sensitivity do not reflect differences in sensitivity of the enzymes themselves. Unfortunately, it proved impossible to estimate the sensitivity of the NAD(P)H oxidase to p-CMB in the absence of any possible permeability barriers since rupture of the cells led to complete loss of this activity.

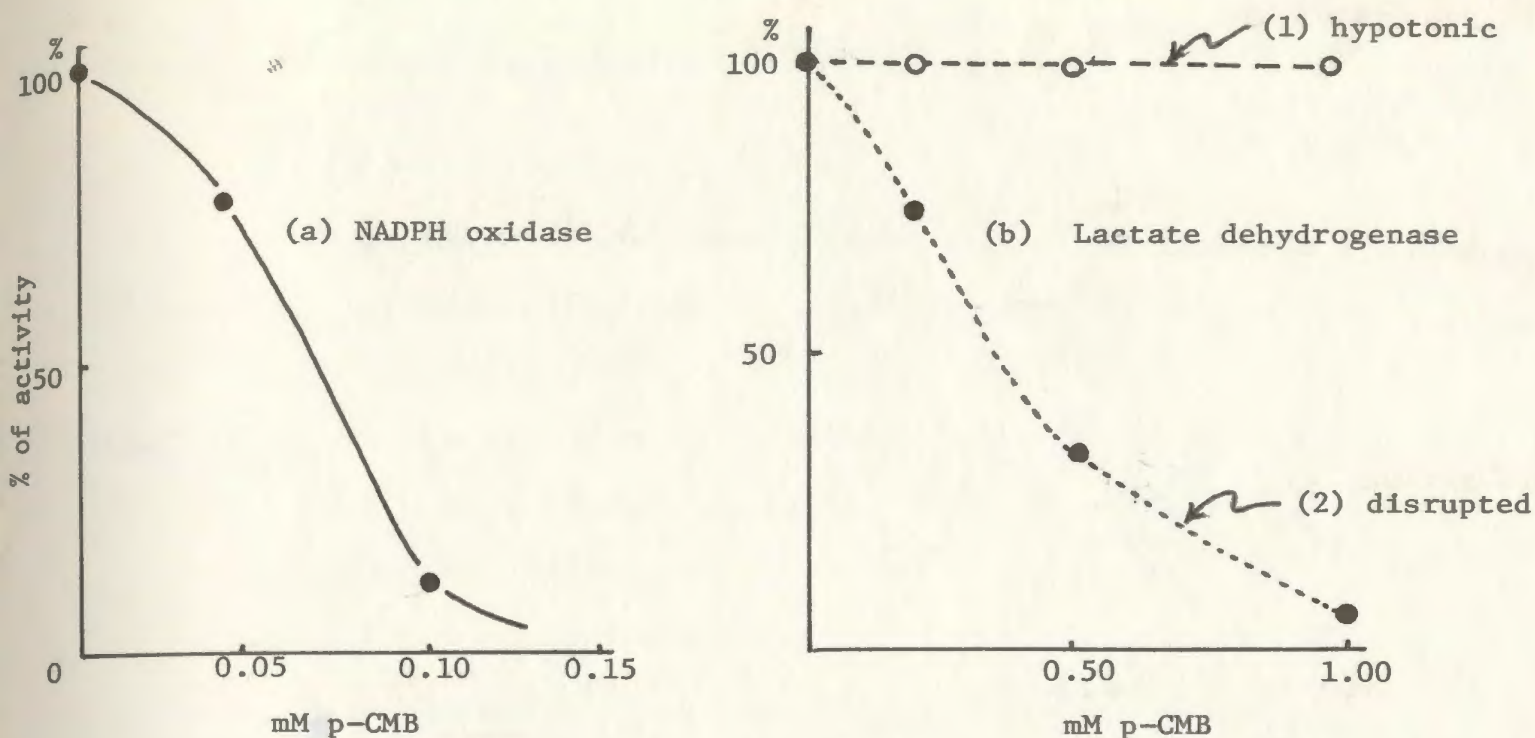


FIGURE 4

Effect of p-CMB on NAD(P)H oxidase and lactate dehydrogenase.

- a) NAD(P)H oxidase activity was measured under the same assay condition as shown in Figure 2. Varied concentrations of p-CMB were added before leukocytes were introduced into the reaction mixture.
- b) Lactate dehydrogenase activity was assayed in the presence of different concentrations of p-CMB.

- (1) --○-- : First, leukocytes (1.3 mg protein) were treated with p-CMB for 5 minutes at 37°C in the hypotonic NAD(P)H oxidase assay system, and then using a 1/10 leukocytes suspension, lactate dehydrogenase was measured with Triton X-100 (final concentration 0.1%).
- (2)●.... : Leukocytes (1.3 mg protein) were disrupted by Triton X-100 (final concentration 0.1%) and lactate dehydrogenase was assayed in the presence of p-CMB.

4. Reversibility of the hypotonic activation of NAD(P)H oxidase

Oxygen uptake was greatly stimulated on incubation of leukocytes in the hypotonic medium in the presence of NADH or NADPH. After two to three minutes, the rate of oxygen uptake began to decrease gradually. Addition of 10% NaCl to the hypotonic medium to make the medium isotonic, decreased the rate of oxygen uptake to that shown normally in an isotonic medium (Figure 5). This reversibility was observed even if the leukocytes were pre-incubated in the hypotonic medium for 10 minutes before addition of NADPH.

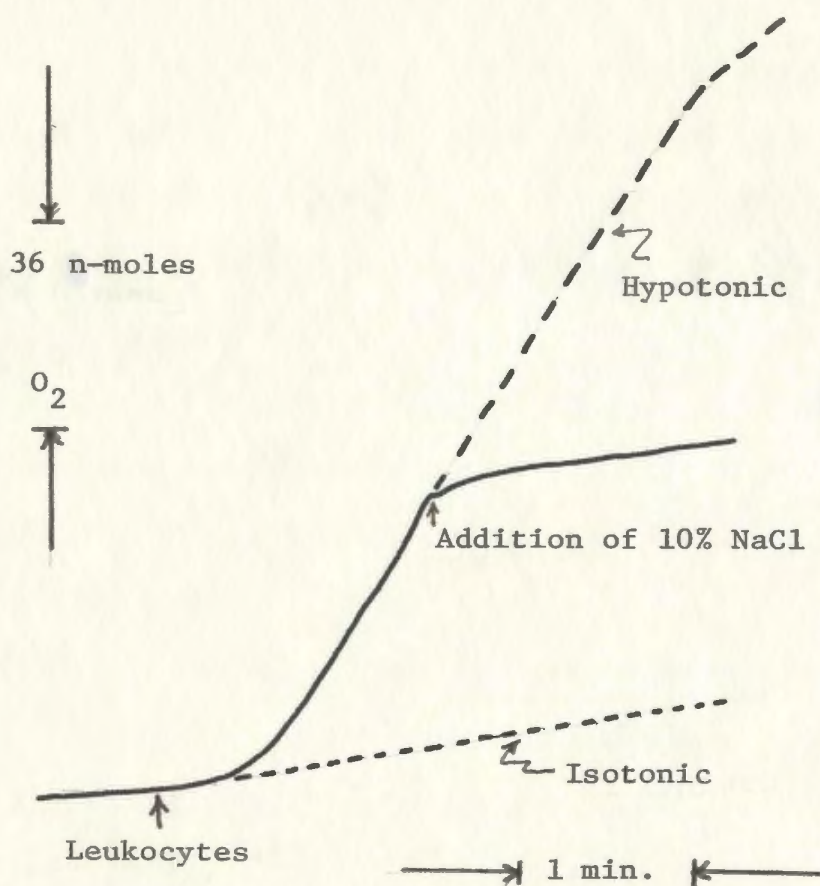


FIGURE 5

Reversibility of the hypotonic activation of NADPH oxidase activity. Experimental conditions were the same as in Figure 2. The solid line represents the activation and the inactivation of NADPH oxidase depending upon the NaCl concentration. 0.15 ml of 10% NaCl was added at the indicated time.

5. Kinetic properties

Complex kinetics were observed for the NAD(P)H oxidase activities in leukocytes when NAD(P)H was the variable substrate. As shown in Figure 6, a biphasic curve for NAD(P)H oxidase was observed between an NADPH concentration of 0.5 mM and 1.0 mM. Similar results were obtained when NADH was used. The K_m values for NADPH and NADH oxidase under hypotonic conditions were approximately 0.5 mM and 1.2 mM, respectively, when calculated from the early portion of the curve. Addition of 2 mM NADH to the reaction medium containing saturating levels (2 mM) of NADPH yielded no further increase in the rate of oxygen uptake. As summarized in Table IV, the hypotonic-treated leukocytes showed a V_{max} of 60-70 n-moles/mg protein/minute for both NADH and NADPH systems.

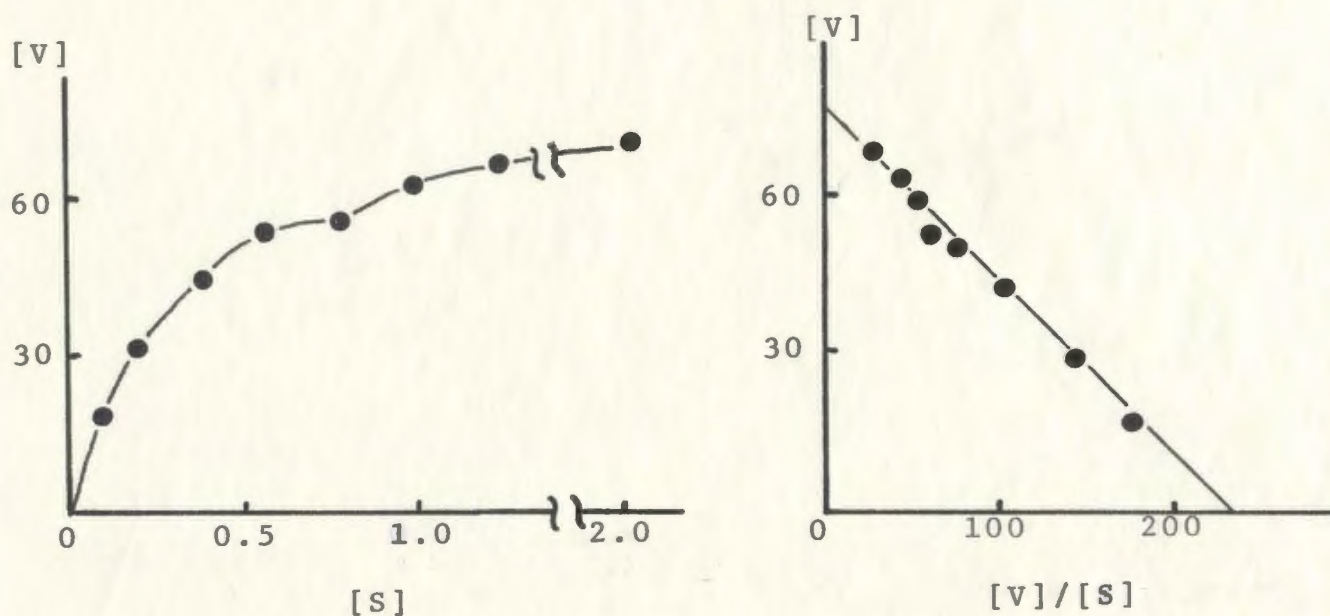


FIGURE 6

Kinetic properties of oxidation of NADPH

The NADPH oxidation reaction was measured with the oxygen electrode in a 1.5 ml vessel containing 60 mM phosphate buffer (pH 6.0), 0.09% NaCl, 1.3 mg leukocyte protein, 2 mM KCN, 0.05 mM MnCl_2 , and varying concentrations of NADPH. [S] represents mM NADPH. [V] is expressed as n-moles/ mg leukocyte protein/min.

TABLE IV

Kinetic Data for NAD(P)H oxidase a)

	NADPH	NADH
K_m b)	0.5 ± 0.2	1.2 ± 0.5
V_{max} c)	72 ± 7	61 ± 15

a) Data is average of three experiments (\pm the range of values)

b) K_m values are expressed as mM.

c) V_{max} are expressed as n-moles/mg protein/min.

DISCUSSION

The results presented in this chapter show that guinea pig leukocytes produce hydrogen peroxide as the principal product of the oxidation of NADH or NADPH in hypotonic medium. A quantity of hydrogen peroxide equivalent to between 50-70% of the oxygen consumed was formed in the presence of cyanide. This yield may be low due to incomplete inhibition of intracellular catalase by cyanide. In later experiments it will be shown that in the presence of 2,4-DCP, over 90% of the oxygen consumed can be accounted for as hydrogen peroxide. It is well established that both oxygen uptake and hydrogen peroxide production by leukocytes are stimulated during phagocytosis (3, 4). Table II demonstrates a stimulation of these processes during hypotonic treatment. The exogenous hydrogen donor required for this process may be either NADH or NADPH. Since the activities of the cytoplasmic dehydrogenases were still largely latent under the hypotonic conditions, it seems likely that the penetration of the pyridine nucleotides through the plasma membrane is still greatly restricted. However, the NAD(P)H oxidase activity was greatly stimulated. This is consistent with NAD(P)H oxidase being located in the plasma membrane.

As a working hypothesis, it is postulated that the phagocytizing process of leukocytes causes conformational changes in the plasma membrane during particle engulfment. Using 8-ANS, Rossi's group (37) has provided direct evidence

for such an event. Since hypotonic conditions also cause a stimulation of oxygen uptake and of NAD(P)H oxidase activity, it seems reasonable to postulate that these occur as a result of similar conformational changes in the plasma membrane. The p-CMB inhibition studies (Figure 3) present further evidence indicating that the NAD(P)H oxidase may be located in the plasma membrane of leukocytes, since the NAD(P)H oxidase activity is inhibited by low concentrations of p-CMB, and p-CMB does not penetrate the plasma membrane.

Rossi et al. speculated that modification of the leukocyte plasma membrane results in the release of an intracellular chemical mediator which stimulates the NADPH oxidase of the granules (37). As Figure 4 demonstrates, however, this assumption seems unlikely since NAD(P)H oxidase activated as a result of hypotonic treatment is immediately inhibited on restoration of the isotonic conditions. Furthermore, most of the work dealing with NADH or NADPH oxidases of leukocytes has been carried out using broken cell preparations. These investigations may not be relevant to a study of hydrogen peroxide formation in intact leukocytes since in our studies the disruption of the cell by either detergent or sonication causes an inactivation of NAD(P)H oxidase activity. Either the intactness of the cell membrane is necessary for H_2O_2 formation or an inhibitor is released from the leukocyte when the membrane is disrupted.

Further evidence demonstrating the resemblance of the NAD(P)H oxidase of hypotonically treated leukocytes to the

NADPH oxidase of granule fractions of phagocytizing leukocytes reported by other workers (27) comes from kinetic studies which show similar K_m values for NAD(P)H in both cases. Rossi's group have shown that the K_m of their oxidase for NADPH decreases ten fold to 4×10^{-4} mM during phagocytosis (29). There is the possibility that the NADH oxidase and NADPH oxidase observed in the cytosol and granules, respectively, by other researchers (33, 54) could be due to contamination by the plasma membrane as a result of homogenization.

In addition, the ability of the plasma membrane-located oxidases to accept NADPH and NADH may help to resolve the controversy between Rossi's group and Karnovsky's group as to whether NADPH or NADH oxidase is the intraleukocytic enzyme for hydrogen peroxide formation during phagocytosis. The NADH oxidase resembles NADPH oxidase with regard to V_{max} , lag period of activation, and the percentage of hydrogen peroxide formed in the oxygen consumption process.

CHAPTER III: THE BURST OF OXYGEN CONSUMPTION IN DIGITONIN TREATED LEUKOCYTES

The antimicrobial action of leukocytes during phagocytosis is associated with an increased uptake of oxygen which is required for H_2O_2 production. When particles make contact with the leukocytes, the cyanide-resistant oxygen uptake is initiated within a few seconds (34). A similar burst of oxygen uptake was also observed when leukocytes were treated with some detergents such as deoxycholate, endotoxin, digitonin (55), saponin (32) or antibodies (56).

In this chapter experiments are described in which the treatment of leukocytes with digitonin was used to investigate the site of hydrogen peroxide production.

MATERIALS AND METHODS

Assay for oxygen consumption

Digitonin (Calbiochem) was suspended in 0.9% NaCl solution and sonicated until the solution was clear. To measure oxygen consumption, a Clark-oxygen electrode with a 1.5 ml vessel was employed. The reactions were carried out at 37°C with 1 mg leukocyte protein in an incubation medium containing 20 mM phosphate buffer (pH 7.2), 0.9% NaCl, 0.1 mM $MgCl_2$, 2 mM KCN and varying amounts of digitonin (optimal concentration of digitonin was 20 μ g/mg leukocyte protein).

Assay for superoxide radicals

To test for the formation of superoxide radicals ($O_2^{\cdot-}$), 50 μ M cytochrome c was added to the above incubation medium and the effect of superoxide-dismutase on the rate of reduction of cytochrome c was determined by measuring the absorbance at 550 nm. An extinction coefficient of 18.7 $cm^{-1}mM^{-1}$ was used in the calculations. Purified superoxide-dismutase was purchased from Pentex.

RESULTS

1. Stimulation of oxygen uptake by digitonin treatment

As shown in Figure 7, a small amount of digitonin causes an increased oxygen uptake in intact leukocytes. The optimal amount of digitonin ($20\text{ }\mu\text{g/mg}$ leukocyte protein) results in a ten fold increase in cyanide-resistant oxygen consumption. With this treatment, on the other hand, the intracellular enzymes lactate dehydrogenase for example, were still largely latent in the leukocytes.

When leukocytes were treated with higher concentrations of digitonin the lactate dehydrogenase lost its latency due either to the disruption of the plasma membrane or to increased substrate permeation into the cell. The trypan blue exclusion test provided further evidence that the optimal amount of digitonin did not disrupt leukocytes. The leukocytes stained with the trypan blue only at higher concentrations of digitonin. This higher concentration of digitonin markedly inhibited the burst of oxygen consumption. This indicates that some changes in the plasma membrane of the leukocyte results in the oxygen burst only when cells are relatively intact. That is, when the plasma membrane is modified by either phagocytosis, surface activators or hypotonic conditions, a stimulation of oxygen uptake may occur.

FIGURE 7

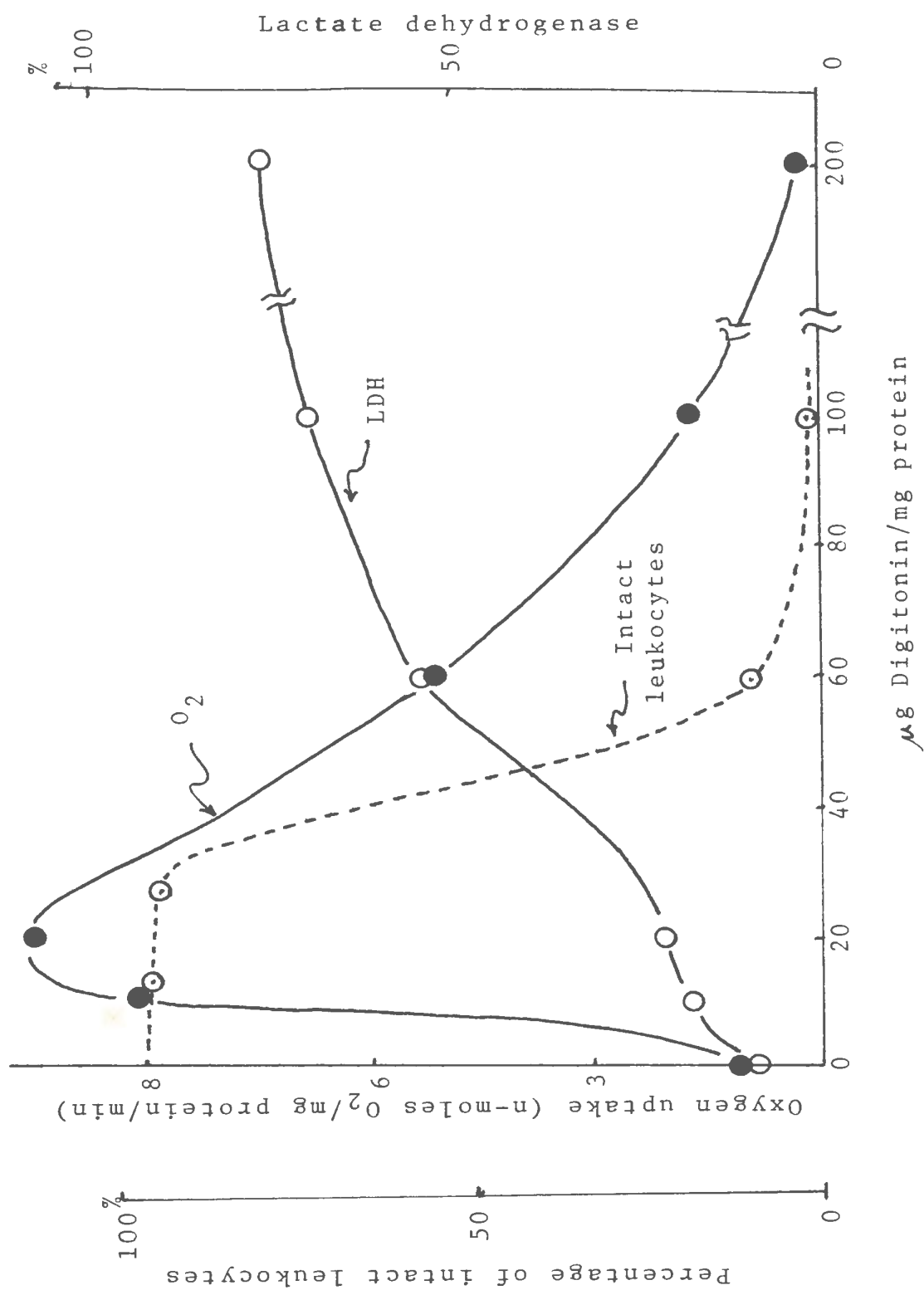


Figure 7: Effects of Digitonin Treatment on Leukocytic
Respiration and Latency of Lactate Dehydrogenase

Oxygen consumption was estimated in a reaction mixture containing 1 mg leukocyte protein, 0.9% NaCl, 20 mM phosphate buffer (pH 7.2), 0.1 mM $MgCl_2$, 2 mM KCN and varied amounts of digitonin in a final volume of 1.5 ml. Incubations were carried out for 5-10 minutes and the maximum rates of oxygen uptake were measured.

Activities of lactate dehydrogenase were assayed using 0.1 ml of the above mentioned reaction mixture after 5 minutes treatment of the leukocytes with various amounts of digitonin. The leukocytes disrupted by 0.1% Triton X-100 were used to calculate the total activity of lactate dehydrogenase. The total activity of lactate dehydrogenase was 1.2 μ moles/min/mg protein. The activity of the lactate dehydrogenase in the digitonin treated leukocytes is expressed as per cent of the total activity.

The percentage of intact leukocytes was measured by the trypan blue exclusion test. Leukocytes were treated with digitonin under the same conditions as in the oxygen consumption experiment. After 2 minutes incubation, 0.1 ml of the suspension was mixed with 0.2 ml trypan blue (0.1%) in 0.9% NaCl and examined under a microscope.

2. Production of superoxide radicals during the burst of oxygen consumption

Using the identical conditions employed in the oxygen consumption experiments described in this chapter, the reduction of oxidized cytochrome c was followed spectrophotometrically. As shown in Figure 8(a), the reduction of cytochrome c by intact leukocytes was greatly stimulated by digitonin treatment. The optimum quantity of digitonin for cytochrome c reduction was 20 μ g per mg protein. This quantity was also optimal for the burst of oxygen consumption (Figure 7). In both cases, higher concentrations were less effective. The reduction of cytochrome c was inhibited by the presence of superoxide dismutase [Figure 8(b)]. Similar phenomena were observed using the oxidation of epinephrine to adrenochrome as an assay for superoxide radicals (data not given). As Yamazaki and co-workers suggested, superoxide radicals may be formed during respiratory burst (57, 58).

FIGURE 8

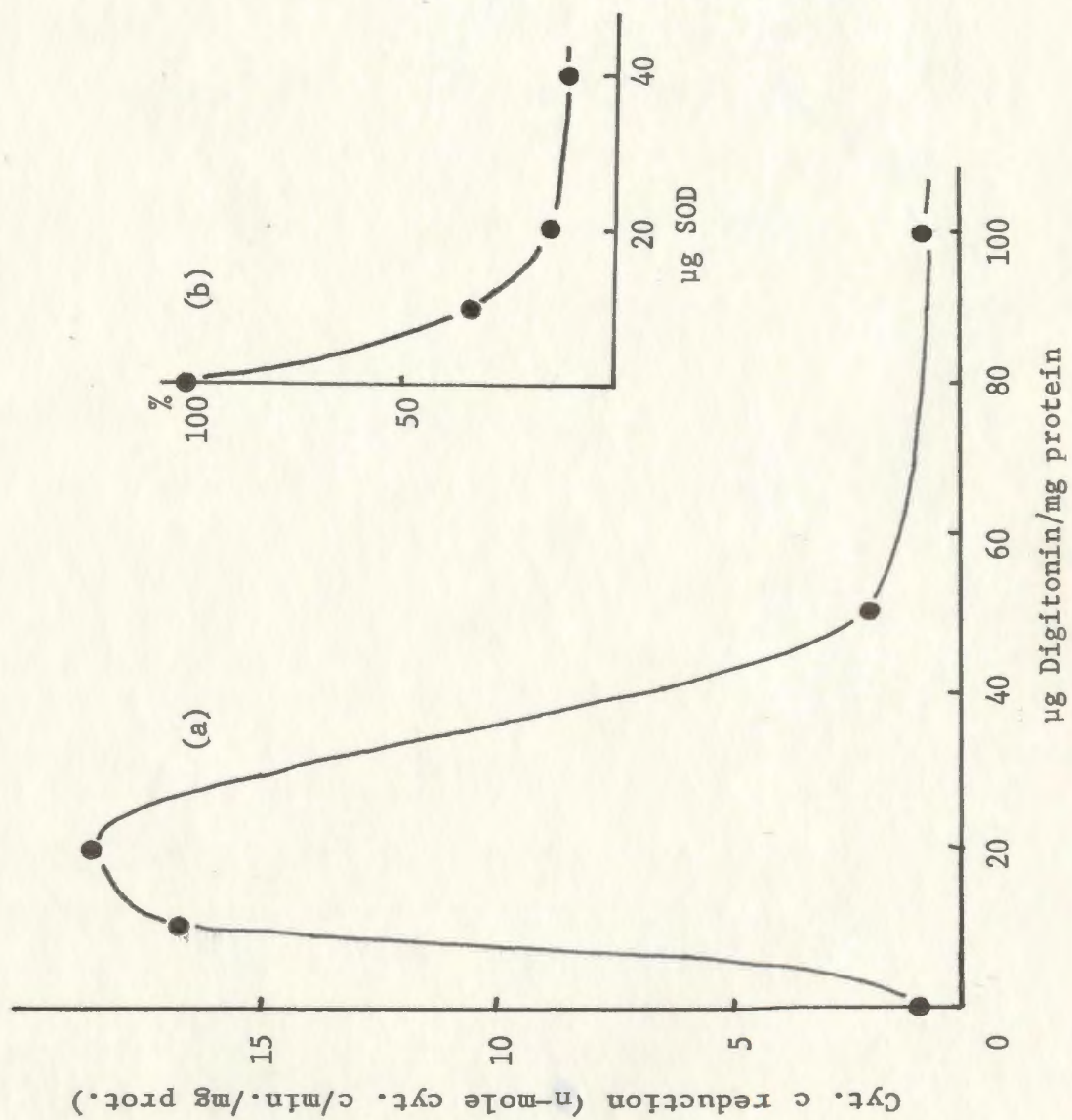


Figure 8: Reduction of Cytochrome c by Digitonin Treated Leukocytes and its Inhibition by Superoxide Dismutase

- (a) This assay was carried out exactly as described in the legend to Figure 7, except that, in addition, 50 μ M cytochrome c was present. The rates of cytochrome c reduction were measured spectrophotometrically at 550 nm. The rates of the reaction were obtained using the linear portion of absorbance increase after the initial lag period.
- (b) Inhibition of the cytochrome c reduction by superoxide dismutase (SOD) was determined in a medium containing leukocytes treated with the optimal amount of digitonin (20 μ g/mg protein of leukocytes).

The dismutase was introduced before the digitonin was added to the reaction mixture.

Results are expressed as per cent activity obtained against the amount of the superoxide dismutase added.

DISCUSSION

Babior et al. have recently observed that phagocytizing leukocytes generate superoxide radicals (12). However, the sites and mechanisms of the superoxide radical generation system in leukocytes have not been clarified. In the previous chapter, it was postulated that NAD(P)H oxidase is located in the plasma membrane of leukocytes. The digitonin treatment of leukocytes provides further evidence, in agreement with the postulate in the previous chapter, that a conformational change in the plasma membrane may initiate the burst of oxygen consumption. Evidence presented in this chapter is summarized as follows:

(a) A striking stimulation of oxygen consumption occurred in the presence of low concentrations of digitonin, presumably as a result of interaction with the plasma membrane.

(b) Disruption of the leukocytes by high concentrations of digitonin was accompanied by complete inhibition of this increased oxygen consumption.

(c) There was a marked stimulation of superoxide production by leukocytes treated with low concentrations of digitonin but not with higher concentrations. The superoxide appeared extracellularly as the plasma membrane is impermeable to superoxide dismutase (MW. 34,000) and cytochrome c (MW. 12,500).

Since the results indicate that the superoxide radical occurs extracellularly, it seems unlikely that the re-

spiratory burst in the digitonin treated leukocytes occurs intracellularly. From these experimental observations it is reasonable to suggest that the key enzyme(s) responsible for the stimulation of oxygen uptake associated with phagocytosis is (are) located in the plasma membrane of the leukocytes. As will be seen later superoxide formation is involved in H_2O_2 formation. The mechanism of superoxide production may involve the NAD(P)H oxidase of the plasma membrane, activated by digitonin modification of the plasma membrane.

CHAPTER IV: EFFECTS OF DICHLOROPHENOL ON RESPIRATION OF LEUKOCYTES

In previous chapters, it was postulated that the burst of oxygen consumption and of hydrogen peroxide and superoxide radical formation by leukocytes was caused by the activation of a plasma membrane associated NAD(P)H oxidase. However, the properties of the NAD(P)H oxidase have not been examined thoroughly. In the present chapter, experiments are reported that bear on the nature of this enzyme. Furthermore, it is demonstrated that the burst of oxygen consumption is markedly stimulated by dichlorophenol. This effect of dichlorophenol on leukocytes is similar to its well-known stimulatory effects on myeloperoxidase.

MATERIALS AND METHODS

The intraperitoneal exudate of guinea pigs containing over 90% polymorphonuclear leukocytes was collected according to the method described in chapter II. In the experiment measuring the oxygen consumption induced by either phagocytosis, detergents, 2,4-dichlorophenol (2,4-DCP) or phospholipase C treatment, Krebs-Ringer phosphate solution (pH 7.4) was used as a washing and incubation medium. For the measurement of NAD(P)H oxidase activity, leukocytes were collected and washed with 0.9%

NaCl solution to eliminate the effects of externally added ions.

Oxygen consumption of leukocytes

Oxygen consumption was measured by a Clark oxygen electrode with a 1.5 ml vessel at 37°C. After 5 minutes preincubation of leukocytes (1.5 mg protein) with or without 2 mM KCN, optimal amounts of materials such as E. coli killed by heating at 100°C for 20 minutes (1 mg protein) (3), paraffin oil emulsion (59), phospholipase C (0.5 units) (28), digitonin (20 μ g) or 2,4-dichlorophenol (1 mM) were introduced into the reaction vessel of the oxygen electrode. (When KCN was added, 20 μ l of 150 mM neutralized KCN solution was used).

RESULTS

1. Stimulation of oxygen consumption by phagocytosis and by dichlorophenol

As summarized in Table V, 2 mM cyanide-resistant oxygen consumption is increased five to ten fold during phagocytosis of either heat-killed E. coli or of paraffin oil emulsion. Both phospholipase C and digitonin treatment of leukocytes also stimulate the oxygen consumption. The increased oxygen uptake commences after a lag period of 30-60 seconds and continues linearly for over 10 minutes.

Furthermore, the addition of 2,4-dichlorophenol (1 mM) to a leukocyte suspension initiates the respiratory burst in the same fashion. The activations of E. coli and 2,4-dichlorophenol are not additive in combination. This indicates that oxygen consumption in the presence of 2,4-DCP and oxygen consumption associated with phagocytosis may occur via a common mechanism. The stimulation of oxygen consumption by 2,4-dichlorophenol was maximal at a concentration of 1 mM (Figure 9). The stimulation of oxygen consumption is not inhibited by 2 mM KCN although the mitochondrial respiration of leukocytes might be inhibited by the KCN.

TABLE V:

Oxygen consumption of leukocytes a)

TREATMENT	2 mM KCN	Absence of KCN
None	5.7	21.0
Killed <u>E. coli</u>	42.1	66.6
Paraffin Oil Emulsion	43.5	- b)
Phospholipase C	30.3	-
Digitonin	45.0	-
2,4-dichlorophenol	40.2	67.3
2,4-DCP + <u>E. coli</u>	57.0	-

a) Respiratory activities of leukocytes (1.5 mg protein) were measured by the oxygen electrode at 37°C. The maximum rate of oxygen consumption was expressed as n-moles O₂/mg leukocyte protein/5 minutes.

b) not measured.

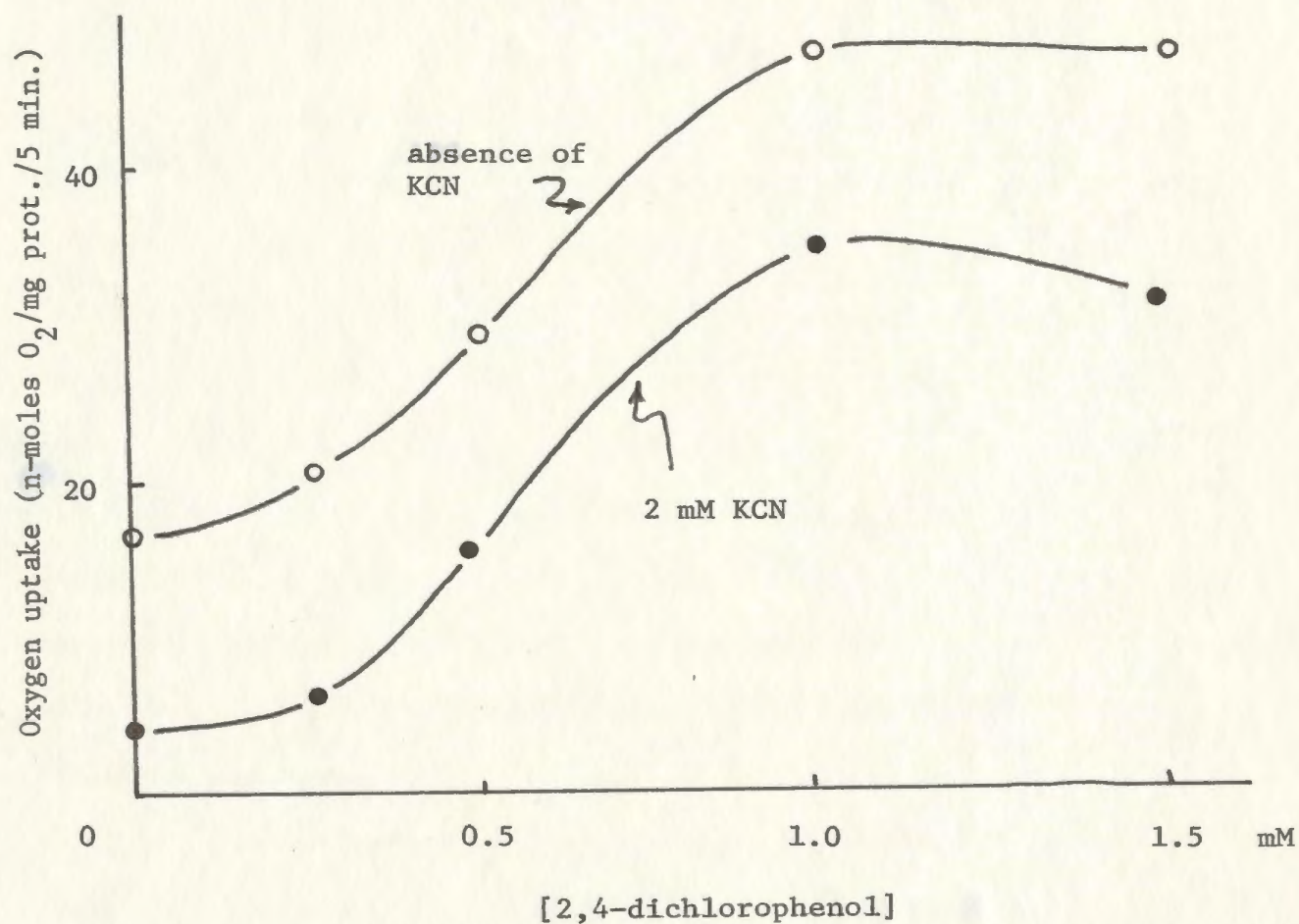


FIGURE 9

Stimulation of oxygen consumption in leukocytes by 2,4-dichlorophenol.

Oxygen consumption was measured as described in the legend to

Table V.

2. Stimulation of NAD(P)H oxidase by dichlorophenol

In the presence of 2 mM KCN and 2,4-DCP, a remarkable burst of oxygen consumption is observed when intact leukocytes are incubated with added NAD(P)H under isotonic conditions (Figure 10). The addition of catalase at the end of oxygen consumption resulted in the liberation of oxygen equivalent to approximately one-half the amount of oxygen consumed. Further addition of NAD(P)H, before the introduction of catalase, gave a further increased oxygen uptake indicating that lack of NADH was the cause of the decreasing rate of oxygen uptake. Spectrophotometric and polarographic measurement of NADH oxidase gave essentially similar results (Table VI). Results listed in Table VI demonstrate the requirements for measuring 2,4-DCP stimulated NADH oxidase activity. The complete system required Mn^{2+} , KCN, 2,4-DCP, NADH and leukocytes.

Increased NADH oxidation was not observed with the sonicated leukocytes under the same experimental condition as intact leukocytes (Table VII). However, addition of H_2O_2 markedly stimulated the activity of sonicated leukocytes. The stimulated activity was about three fold greater than the activity in the intact leukocytes under similar conditions.

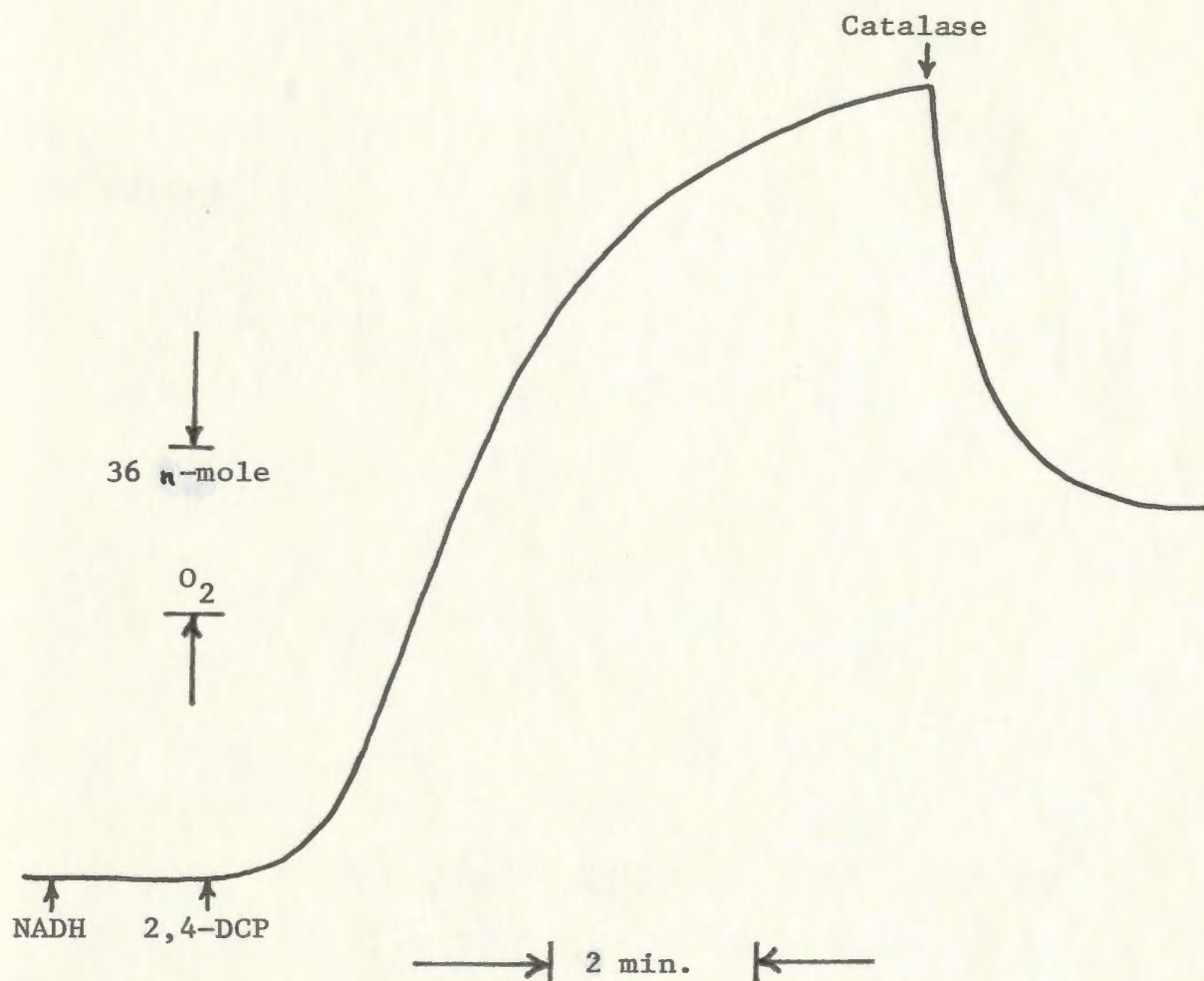


FIGURE 10:

Record of the uptake of oxygen during NADH oxidation and the liberation of H_2O_2 accumulated by intact polymorphonuclear leukocytes (0.15 mg protein).

The reaction medium was as follows: 0.9% NaCl, 50 mM phosphate buffer (pH 6.0), 0.1 mM NADH, 0.5 mM $MnCl_2$, 2 mM KCN, and 1 mM 2,4-dichlorophenol (2,4-DCP). When catalase was added, 35×10^3 units were used.

TABLE VI: Requirements for the 2,4-dichlorophenol-stimulated NADH oxidase reaction

EXPERIMENT NUMBER	CONDITIONS	O ₂	NADH (measured at 340 nm)
1	Complete	262.0	166.5
2	Omit MnCl ₂	76.2	53.8
3	Omit KCN	84.6	11.7
4	Omit 2,4-DCP	10	10
5	Omit NADH	10	-
6	Omit leukocytes	10	10

The complete reaction mixture contained 0.15 mg protein of PMNs, 0.5 mM MnCl₂, 0.1 mM NADH, 2 mM KCN and 1 mM 2,4-dichlorophenol (2,4-DCP) in 50 mM phosphate buffer (pH 6.0) in 0.9% NaCl at 37°C. Final volume was 1.5 ml. The reaction was initiated by the addition of 2,4-dichlorophenol. The blank reaction contained no leukocytes. Results were calculated from the maximum rates as n-moles consumed/mg protein/minute.

TABLE VII: Effect of Various Factors on the 2,4-Dichlorophenol Stimulated NADH Oxidase ^{a)}

CONDITIONS ^{b)}	INTACT	SONICATED ^{b)}
NADH	10	10
NADH + KCN	10	10
NADH + KCN + 2,4-DCP	42.6	10
NADH + KCN + Mn ²⁺ + 2,4-DCP	129.0	10
NADH + KCN + Mn ²⁺ + 2,4-DCP + H ₂ O ₂ ^{c)}	122.8	337.8
NADH + KCN + 2,4-DCP + H ₂ O ₂	43.5	155.0

a) The reaction medium was the same as in Table VI. The initial rate of NADH oxidation was expressed as n-moles NADH oxidized/min./mg protein measured spectrophotometrically.

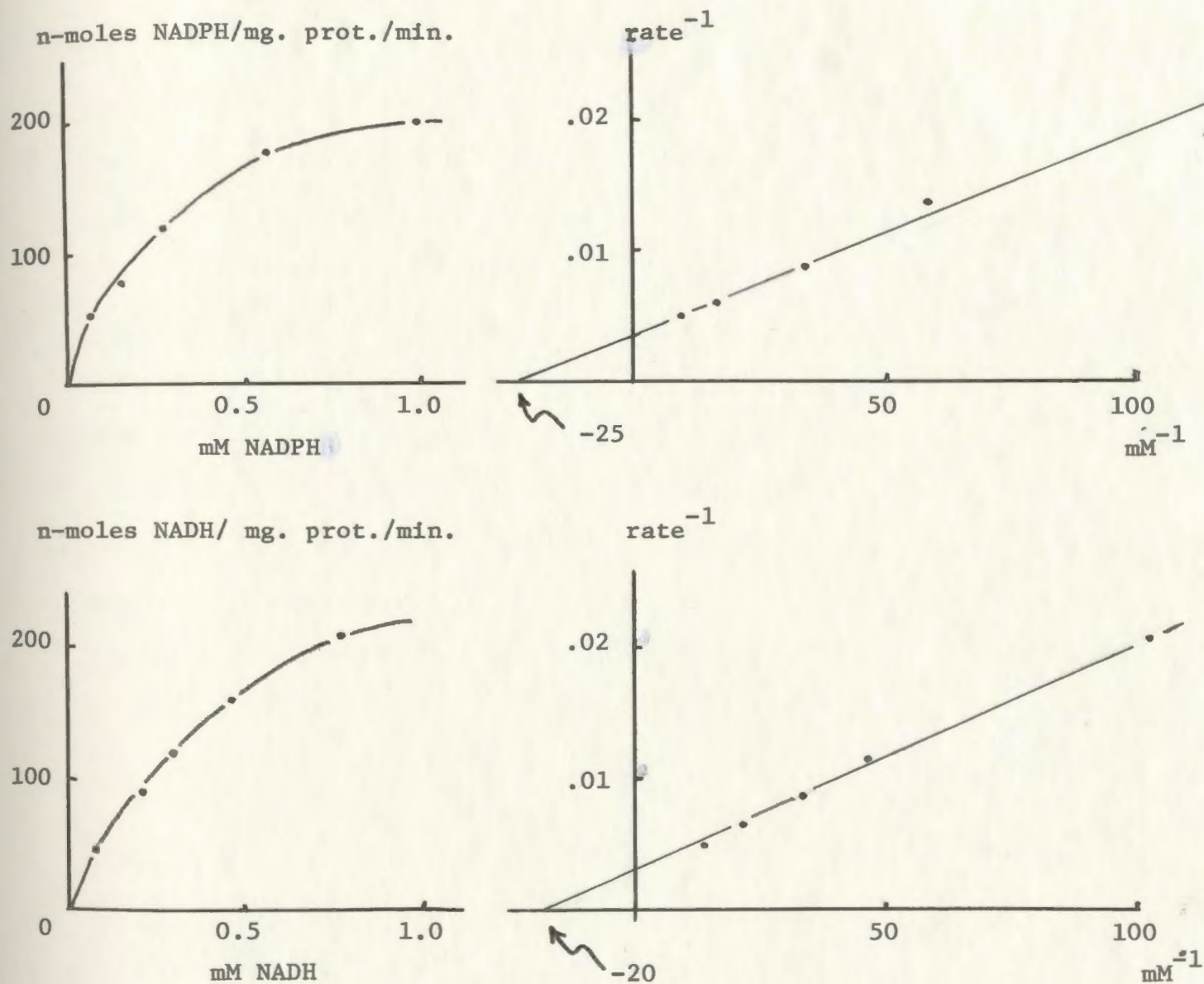
b) Suspended leukocytes (10 mg/ml) were sonicated (50 watts for 60 seconds) at 0°C.

c) 0.1 μ mole H₂O₂ was introduced into the assay system.

3. Kinetic studies of the dichlorophenol activated NAD(P)H oxidase of intact cells

The enzymatic oxidation of the reduced pyridine nucleotides by intact leukocytes was linear as a function of time for a period of 1 to 5 minutes, although there was a small initial lag period. Therefore, the reaction rate after this lag period was measured as a function of NAD(P)H concentration. From the Lineweaver-Burk plots, (Fig 11) the K_m values of NADH and NADPH were calculated to be 50 μM and 40 μM , respectively. The V_{max} values of these reactions were approximately 300 n-moles NAD(P)H/mg protein of leukocytes/min in both cases.

FIGURE 11: Kinetics of the Oxidation of NADH and NADPH



Details of the experimental conditions are described in the methods section of this chapter.

4. Effect of dichlorophenol on the latency of leukocyte enzymes

The enzymatic oxidation of guaiacol by peroxidase in intact leukocytes has a very low activity as shown in Table VIII. Sonication enhances the activity more than fifteen times. The dehydrogenases, such as malate dehydrogenase and lactate dehydrogenase, were also activated when leukocytes were broken by sonication. The addition of KCN, 2,4-dichlorophenol and/or MnCl_2 did not affect the latency of these three enzymes. Furthermore, the addition of low concentrations of p-CMB (0.1 mM) clearly inhibited the NAD(P)H oxidation by the intact leukocytes, whereas cytoplasmic enzyme (lactate dehydrogenase) was not affected (Figure 12). Only when high concentrations of p-CMB (1 mM) were added after disruption of the leukocytes was the lactate dehydrogenase inhibited.

TABLE VIII : Latency of enzyme activities

	Intact	Sonicated
Guaiacol peroxidase a)	183 (6.7%) ^{c)}	2260
Lactate dehydrogenase b)	135 (11.1%) ^{c)}	1220
Malate dehydrogenase b)	19.2 (6.3%) ^{c)}	306

a) Guaiacol peroxidase activities were expressed as the formation of n-moles tetraguaiacol/mg protein/minute.

b) Both dehydrogenase activities were determined by the rate of decrease of absorbance of NADH at 340 m . Results were expressed as n-moles NADH/mg protein/minute.

c) The activity in intact leukocytes is expressed as a percentage of that in sonicated leukocytes.

FIGURE 12: Effect of p-CMB on NAD(P)H oxidase and lactate dehydrogenase

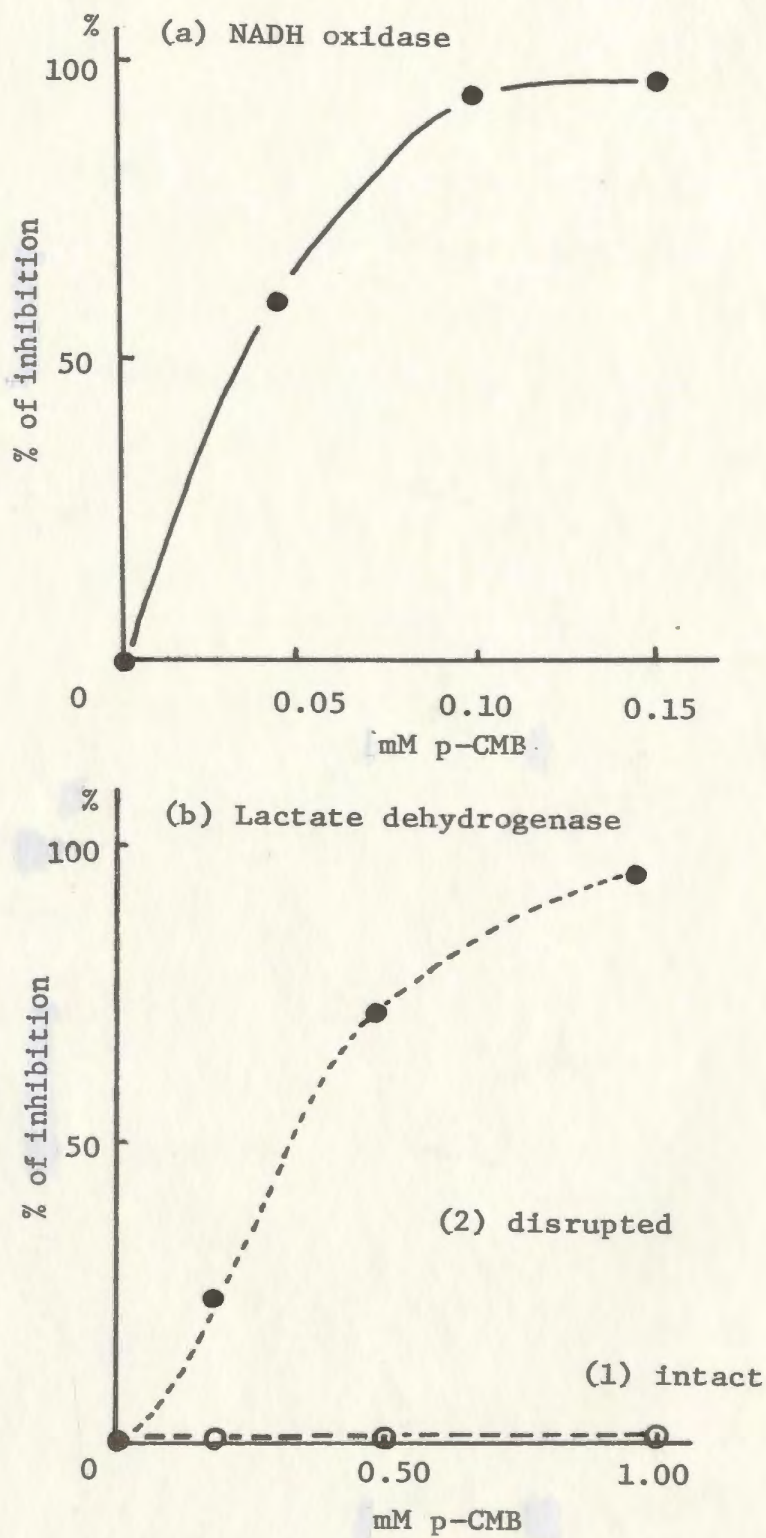


FIGURE 12.

Effect of p-CMB on 2,4-DCP activated NAD(P)H oxidase and lactate dehydrogenase

(a) NAD(P)H oxidase activity was measured spectrophotometrically under the same assay conditions as shown in Table II. Different concentrations of p-CMB were added to the leukocyte suspension and 2 minutes later 2,4-DCP was introduced into the reaction mixture.

(b) Lactate dehydrogenase activities were assayed in the presence of different concentrations of p-CMB.

1. — — o — — : Leukocytes (0.15 mg protein were treated with p-CMB for 2 minutes at 37°C in the medium used for the determination of NADH oxidase. Then lactate dehydrogenase was measured in the presence of Triton X-100 (final concentration of 0.1%).

2. --●--- : Leukocyte (0.15 mg protein) was disrupted by Triton X-100 (final concentration 0.1%) and lactate dehydrogenase was measured in the presence of p-CMB.

DISCUSSION

The results presented in this chapter show that leukocytes produce H_2O_2 as the principal product of the oxidation of NAD(P)H in the presence of 2,4-dichlorophenol and 2 mM KCN. It is well established that 2,4-dichlorophenol is a stimulator of the oxidase-reaction of peroxidase (46, 57). In the presence of Mn^{2+} , purified horseradish peroxidase can oxidize reduced pyridine nucleotides, and the oxidation is markedly stimulated by phenols (46). Although Iyer and Quastel (45) suggested that the myeloperoxidase may play a role as NAD(P)H oxidase in leukocytes, they were unable to propose a precise mechanism. In fact, the myeloperoxidase system was previously considered unlikely to be responsible for H_2O_2 production in leukocytes since it was cyanide sensitive.

In the previous chapter it was demonstrated that a cyanide-resistant NAD(P)H-oxidase activity of intact leukocytes was stimulated by hypotonic treatment with resultant formation of H_2O_2 . In this chapter, further properties of the oxidase believed to be located on the outside surface of the leukocyte and involved in the phagocytosis-induced respiratory burst are discussed.

There are three lines of experimental evidence supporting the hypothesis that a plasma membrane located peroxidase is involved in H_2O_2 formation in the phagocyt-

izing process:

(i) The cyanide-resistant oxygen uptake of leukocytes is also activated by 2,4-dichlorophenol. Dichlorophenol did not affect the integrity of the cell or the permeability of the plasma membrane, as lactate dehydrogenase was still latent and the cells excluded trypan blue.

(ii) The NAD(P)H oxidase activity of intact leukocytes is strongly inhibited by p-CMB which does not permeate the plasma membrane.

(iii) A remarkable NAD(P)H dependent oxygen consumption is observed with intact leukocytes in the presence of 2,4-dichlorophenol, MnCl_2 and KCN at pH 6.0.

It seems reasonable to assume from these experimental observations that the cyanide-resistant NAD(P)H oxidase activity of leukocytes is not due to the intragranular myeloperoxidase because intact leukocytes have a very low guaiacol peroxidase activity which is activated greatly by disruption of the cells, whereas NAD(P)H oxidase activity is fairly high in the intact leukocytes. Further discussion of the NAD(P)H oxidase and a peroxidase will be seen in the next chapter. One mechanism for the oxidase reaction could involve hydrogen peroxide. A small amount of hydrogen peroxide formed by the autoxidation of NAD(P)H in the presence of cyanide could initiate the enzymatic oxidation of NAD(P)H by a peroxidase reaction (46). Whilst the addition of hydrogen peroxide does not cause further stimulation of NAD(P)H

oxidation in intact leukocytes, the reaction replaced sonicated leukocytes in the presence of Mn^{2+} , 2,4-DCP and KCN has a requirement for added H_2O_2 . Presumably the H_2O_2 is involved as a catalyst for the oxidase reaction of the myeloperoxidase. However, a peroxidase reaction could not account for the oxidation of NAD(P)H by the myeloperoxidase as cyanide and Mn^{2+} were present and these are known to inhibit this peroxidase reaction. Furthermore it will be demonstrated in the next chapter that H_2O_2 can stimulate oxygen uptake catalyzed by the purified myeloperoxidase and thus a partial radical chain reaction may be catalyzed by the H_2O_2 . In the presence of added H_2O_2 (0.1 μ mole), the NADH oxidase activity of sonicated leukocytes is three fold greater than that of intact cells. This may be due to the release of granule-bound myeloperoxidase.

As shown in Figure 10, one half of the oxygen consumed by intact leukocytes can be liberated with excess amounts of catalase indicating that most of the utilized oxygen is converted to hydrogen peroxide during the reaction process in the presence of the cyanide. In addition, the NAD(P)H oxidase shows less activity when cyanide is omitted from the reaction mixture (Table VI).

It has been demonstrated by Rossi et al. that the cyanide resistant NADPH oxidase in granule fractions of

phagocytizing leukocytes has no latency. The appearance of NAD(P)H oxidase in the granule fraction or in the cytosol fraction is dependent upon the methods of homogenization and it is probable that the plasma membrane of the leukocyte sediments with the lighter granule fraction in Rossi's preparation. Therefore the oxidase activity of the granule fractions by Rossi would be due to granule-located myeloperoxidase and to plasma membrane located oxidase.

From the observations in this chapter, it seems most likely that the hydrogen peroxide generating enzyme system is in the plasma membrane. This could explain the increased NADPH oxidase observed by Rossi for the granule fraction from phagocytizing leukocytes as H_2O_2 produced at the plasma membrane could have activated the intragranular peroxidase.

CHAPTER V: COMPARATIVE STUDIES ON THE OXIDASE ACTIVITIES OF INTACT LEUKOCYTES AND PURIFIED PEROXIDASES

It is suggested that the leukocytic hydrogen peroxide generating system is associated with the oxidase reaction of a peroxidase, since 2,4-dichlorophenol and Mn^{2+} strongly stimulate the NAD(P)H oxidase activity of leukocytes. Intact leukocytes show low guaiacol peroxidase activity, however, although they have a powerful NAD(P)H oxidase activity. Therefore a comparison of pure horseradish peroxidase, partially purified myeloperoxidase, and intact leukocytes was made to characterize further the mechanism of leukocytic NAD(P)H oxidase.

MATERIALS AND METHODS

Polymorphonuclear leukocytes were collected according to the previously described methods in Chapter 1, washed, and suspended in 0.9% NaCl solution. Peroxidase (horseradish peroxidase, 120 enzyme units/mg) was obtained from SIGMA and dissolved in distilled water (1 mg/10ml).
 * (One unit=Formation of 1 mg Purpurogallin in 20 seconds from Pyrogallol at pH 6.0 at 20°C according to Sigma catalogue).
Procedure for the partial purification of myeloperoxidase

Polymorphonuclear leukocytes were obtained according to the method described in Chapter I, except that 30 ml of NaCl solution containing 500 units of heparin (Connaught Medical Research Laboratories) were employed to wash out the

leukocytes from each guinea pig. The leukocyte suspension was centrifuged (500 x g for 5 min) and washed once with ice cold 0.25 M sucrose. The cells were then resuspended in 10 ml of 0.34 M sucrose and placed in an ice bucket for 15 min. Homogenization was carried out (for 3-4 minutes) with a Potter-type homogenizer with a teflon pestle. The homogenate was centrifuged at 500 x g for 10 minutes to remove unbroken cells and nuclei. The resultant supernatant was centrifuged at 13,000 x g for 15 minutes, the sedimented fraction was resuspended in 0.2 M NaCl (7 ml), the CETABRON (cetyltrimethylammonium bromide) (final concentration 0.05%) was added to solubilize myeloperoxidase and/or NADPH oxidase from the granule fraction (26). The CETABRON-treated granule suspension was centrifuged in the Beckman model L2 ultra-centrifuge at 105,000 x g for 60 minutes to remove the CETABRON insoluble residue.

The resultant supernatant, approximately 5-6 ml, was further purified by the following chromatographic procedures (60). Pharmacia CM-Sephadex (CM-50) was swollen with distilled water for 2 days and washed with 25 mM acetate buffer (pH 4.7) containing 0.2 M NaCl solution. The ion-exchange material was packed in a column (1.2 cm diameter, approximately 30 cm high), and approximately 3 cm of Pharmacia Sephadex G-100 was layered on the top of it. After the enzyme extract was introduced onto the column, elution was performed at 0-4°C with a continuous gradient of NaCl (0.2 to 1.5 M) containing 25 mM acetate buffer (pH 4.7).

5 ml fractions were collected.

Enzyme assays

The oxidation of NAD(P)H was followed by measuring the decrease in light absorption at 340 nm at 37°C under aerobic conditions in a final volume of 3 ml. The assay medium for the oxidase reaction system contained 50 mM phosphate buffer (pH 6.0), 0.1 mM NAD(P)H, 0.5 mM MnCl_2 , 0.9% NaCl and enzyme (peroxidase or leukocytes). The reaction was started by the addition of 20 μl of 150 mM 2,4-dichlorophenol (final concentration of 1.0 mM).

Peroxidase activities were assayed by following the oxidation of NADH at 340 nm at 37°C under anaerobic conditions. The reaction medium (3 ml) contained 0.1 mM NADH, 0.3 mM H_2O_2 , 1 mM 2,4-dichlorophenol, 0.9% NaCl and 50 mM phosphate buffer (pH 6.0). The medium was flushed with oxygen-free N_2 gas for 60 seconds and the enzyme was tipped into the reaction mixture from the side arm of the anaerobic cuvette. The rates, expressed as the decrease in NADH per 1 minute, were calculated from the maximum rates obtained during the first five minutes after enzyme addition.

The rate of reduction of NBT (Nitroblue tetrazolium) with the NAD(P)H oxidase system was assayed under the following conditions (6l). Peroxidase or leukocytes were suspended in a 0.9% NaCl medium containing 0.5 mM NADH or NADPH, 0.5 mM MnCl_2 , 1.0 mM 2,4-DCP, 50 mM NBT and 50 mM

phosphate buffer (pH 6.0). The rate of the reaction was obtained after the initial lag using the linear portion of the absorbance increase at 560 nm.

RESULTS

1. Oxidase and peroxidase reactions of HRP

To compare the oxidase and peroxidase properties of horseradish peroxidase, the 2,4-DCP-stimulated NADH oxidation system was assayed under aerobic and anaerobic conditions. As shown in Table IX, the peroxidase reaction required NADH, 2,4-DCP and H_2O_2 under anaerobic conditions. Low concentrations (0.1 mM) of cyanide inhibited over 90% of this reaction. Addition of manganese ions to the complete system decreased the peroxidase activity, although it stimulated the oxidase reaction strongly.

Using the same concentration of substrates and enzyme as in the peroxidation reaction, the requirements for NADH oxidation were investigated. In the optimal conditions for the NADH oxidase assay system, the reaction medium contained NADH, 2,4-DCP and $MnCl_2$. The addition of hydrogen peroxide (0.3 mM) decreased the NADH oxidizing activity under aerobic conditions.

TABLE IX: OXIDATION OF NADH BY HORSERADISH PEROXIDASE

<u>Anaerobic Condition</u>	
<u>Conditions</u>	<u>Specific Activities</u> ^{a)}
NADH, 2,4-DCP, H_2O_2	100.1
NADH, 2,4-DCP	6.3
NADH, H_2O_2	7.1
NADH, 2,4-DCP, $MnCl_2$	5.2
NADH, 2,4-DCP, H_2O_2 , $MnCl_2$	28.1
NADH, 2,4-DCP, H_2O_2 , KCN (0.1 mM)	6.2
NADH, 2,4-DCP, H_2O_2 , KCN (1.0 mM)	3.3

<u>Aerobic Condition</u>	
<u>Conditions</u>	<u>Specific Activities</u> ^{a)}
NADH, 2,4-DCP, H_2O_2	104.1
NADH, 2,4-DCP, H_2O_2 , KCN (0.1 mM)	48.0
NADH, 2,4-DCP, H_2O_2 , KCN (1.0 mM)	28.2
NADH, 2,4-DCP	106.5
NADH, 2,4-DCP, $MnCl_2$	250.0
NADH, 2,4-DCP, H_2O_2 , $MnCl_2$	78.0
NADH, 2,4-DCP, H_2O_2 , $MnCl_2$, KCN (1.0 mM)	35.0
NADH, H_2O_2	1.5
NADH, H_2O_2 , $MnCl_2$	1.5

a) Specific activities are expressed as n-moles NADH/minute/
enzyme unit of HRP.

2. Oxidase and peroxidase properties of intact leukocytes

The 2,4-DCP-stimulated NADH oxidation system of leukocytes has a very low activity under anaerobic conditions and its activity is increased almost twenty fold when the cells are disrupted by Triton X-100 (Table X). On the other hand, aerobic oxidase reactions show fairly high activity and Triton X-100 treatment of leukocytes results in only two-three fold increase.

TABLE X: OXIDATION OF NADH BY LEUKOCYTES

<u>Anaerobic Condition</u>	
<u>Conditions</u>	<u>Specific Activities</u> ^{a)}
NADH, 2,4-DCP, H_2O_2	9.3
NADH, 2,4-DCP	1.8
NADH, 2,4-DCP, H_2O_2 , KCN(1.0 mM)	0.6
NADH, 2,4-DCP, H_2O_2 , $MnCl_2$	6.3
NADH, H_2O_2	6.3
^{b)} NADH, 2,4-DCP, H_2O_2	163.5

<u>Aerobic Condition</u>	
<u>Conditions</u>	<u>Specific Activities</u> ^{a)}
NADH, 2,4-DCP, $MnCl_2$, KCN(1.0 mM)	228.3
NADH, 2,4-DCP, $MnCl_2$	95.4
NADH, 2,4-DCP	8.7
NADH, 2,4-DCP, H_2O_2 , $MnCl_2$	43.5
NADH, 2,4-DCP, H_2O_2	20.1
^{b)} NADH, 2,4-DCP, $MnCl_2$	714.5
^{b)} NADH, 2,4-DCP, $MnCl_2$, KCN(1.0 mM)	438.7

a) Results are expressed as n-moles NADH/mg leukocyte protein/minute.

b) Leukocytes were disrupted by 0.1% of Triton X-100.

3. Effect of cyanide on the oxidase and peroxidase reaction

As shown in Figure 13, the effect of cyanide on horseradish peroxidase depends upon the reaction measured. The NADH oxidase activity of HRP without 2,4-DCP is not appreciably inhibited by even high concentrations of cyanide, although the 2,4-DCP-stimulated oxidase activity is inhibited. In the well-known guaiacol peroxidase reaction, the activity is almost completely inhibited by much lower (0.1 mM) concentrations of cyanide.

On the other hand, as indicated in Figure 14, intact leukocytes show some curious sensitivity of 2,4-DCP-stimulated NADH oxidase with regard to cyanide concentrations. In the absence of cyanide the leukocyte suspensions have a low NADH oxidizing activity, and the addition of a low concentration of cyanide (0.5 mM) increases its activity. However, higher concentrations of cyanide (1.0 mM) inhibit the NADH oxidase.

FIGURE 13: Effect of Cyanide on Oxidase and Peroxidase Reactions of

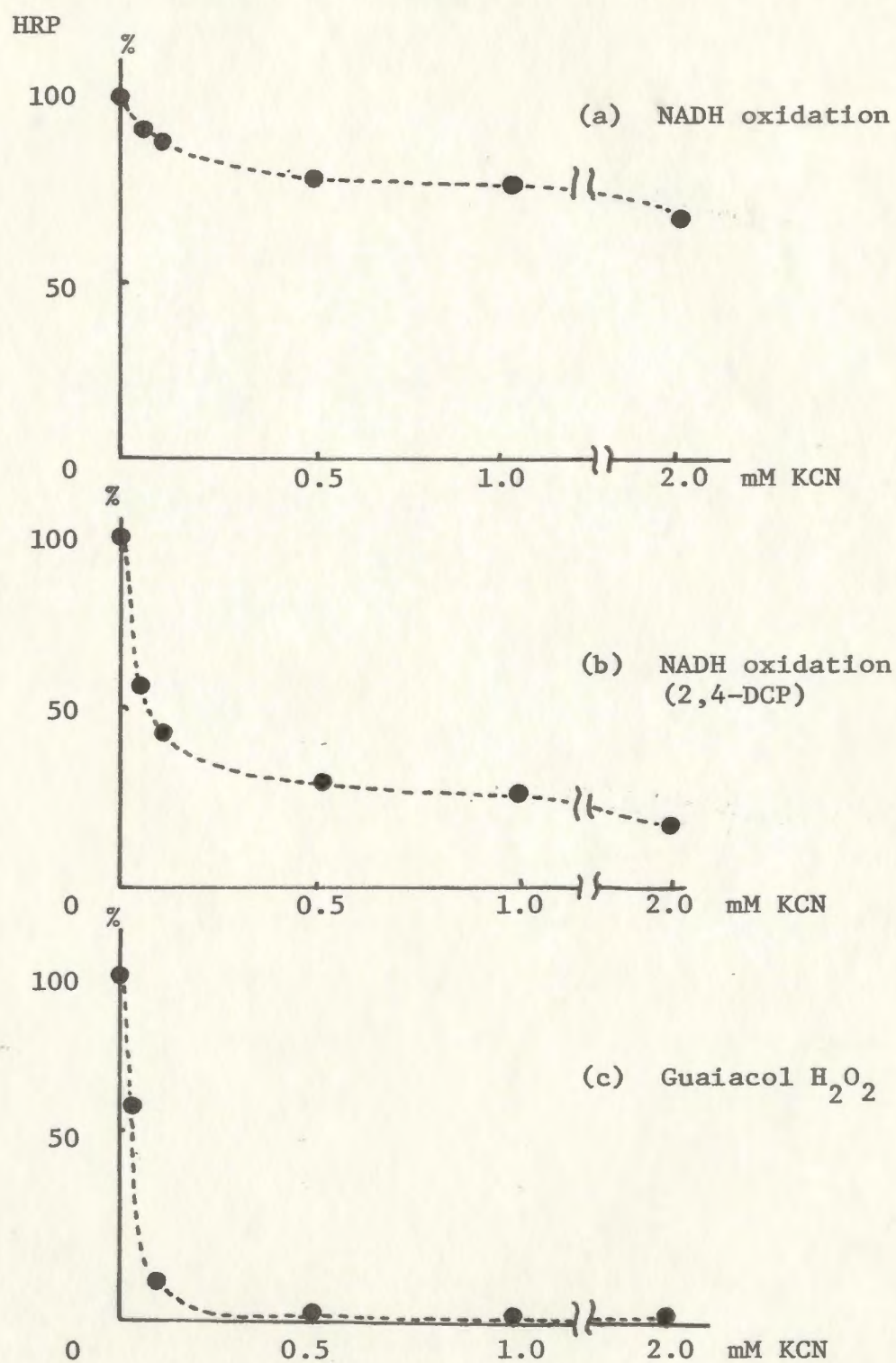
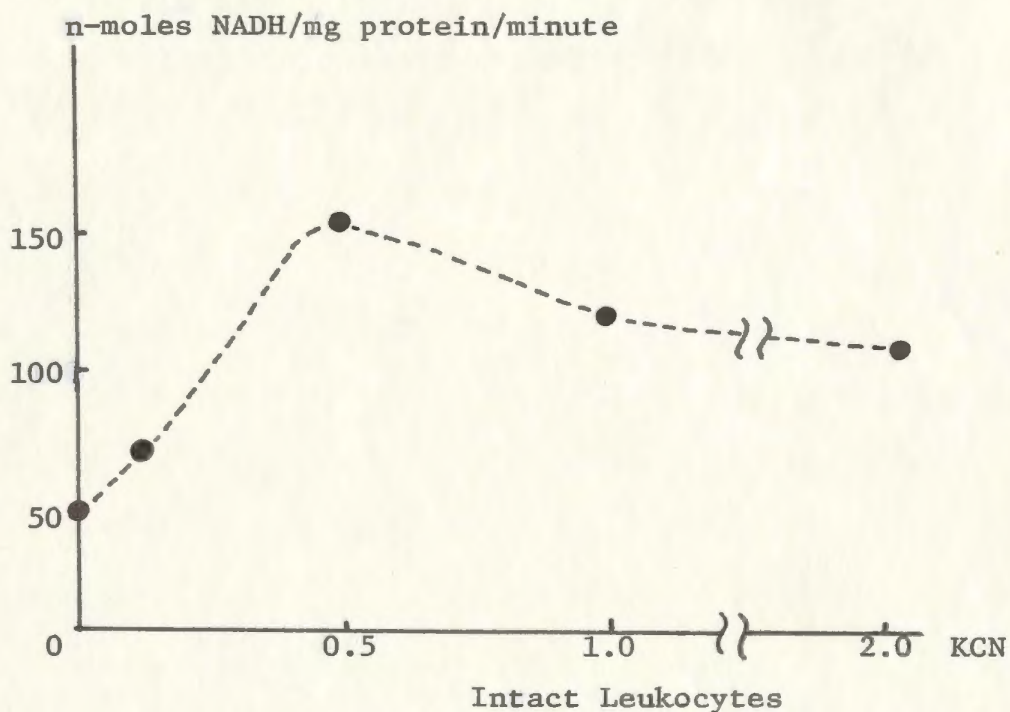


FIGURE 13: Effect of Cyanide on Oxidase and Peroxidase Reactions of HRP

- (a) NADH oxidation: Reaction mixtures contained 1.0 mM NADH, 0.5 mM MnCl_2 , 1.2 units of HRP, 50 mM phosphate buffer (pH 6.0) and varied concentrations of neutralized KCN in total volume 1.5 ml. The reaction was performed under aerobic conditions with the oxygen electrode. The linear portion of the oxygen consumption was measured as the rate. 100% value was 25 n-moles O_2 /minute/enzyme unit.
- (b) NADH oxidation stimulated by 2,4-DCP: Reaction mixtures contained 0.1 mM NADH, 0.5 mM MnCl_2 , 1 mM 2,4-DCP, 0.12 units of HRP, 50 mM phosphate buffer (pH 6.0) and varied concentrations of neutralized KCN in total volume 1.5 ml. The rates were assayed as described above. 100% value was 250 n-moles O_2 /min/enzyme unit.
- (c) Guaiacol-peroxidation: Reaction mixture contained 10 mM guaiacol, 3 mM H_2O_2 , 50 mM phosphate buffer (pH 6.0), 0.12 units of HRP (in the absence of KCN, 0.012 units of HRP was used) and varied concentrations of KCN in a total volume 1.5 ml. 100% value was 2.2μ moles/min/enzyme unit.

FIGURE 14: Effects of Cyanide on the Oxidase Reaction of Leukocytes



The 2,4-DCP-stimulated NADH oxidase activity was assayed in the reaction mixture containing 0.2 mg protein of leukocytes, 0.5 mM MnCl_2 , 1 mM 2,4-DCP, 0.1 mM NADH, 0.9% NaCl, 50 mM phosphate buffer (pH 6.0) and varied amounts of neutralized KCN at 37°C in 1.5 ml. The rate of NADH oxidation was measured by the decrease of absorbance at 340 nm.

4. Properties of partially purified myeloperoxidase as NAD(P)H oxidase

As shown in Table XI, a thirty fold purification of myeloperoxidase (measured as guaiacol peroxidase) was achieved. In the fractions after chromatography, guaiacol peroxidase activity and 2,4-DCP-stimulated NADH oxidase activity appeared in the same peak (data not shown). Although NAD(P)H oxidase activity of the partially purified myeloperoxidase in the absence of 2,4-DCP was very small, addition of a low concentration of hydrogen peroxide greatly enhanced the activity (Figure 15). Similar results were observed when NADH was used as a hydrogen donor instead of NADPH. Furthermore, aminotriazole (which is known to inhibit peroxidases) also stimulated the initial rate of NAD(P)H oxidase activity at least five fold (Figure 16).

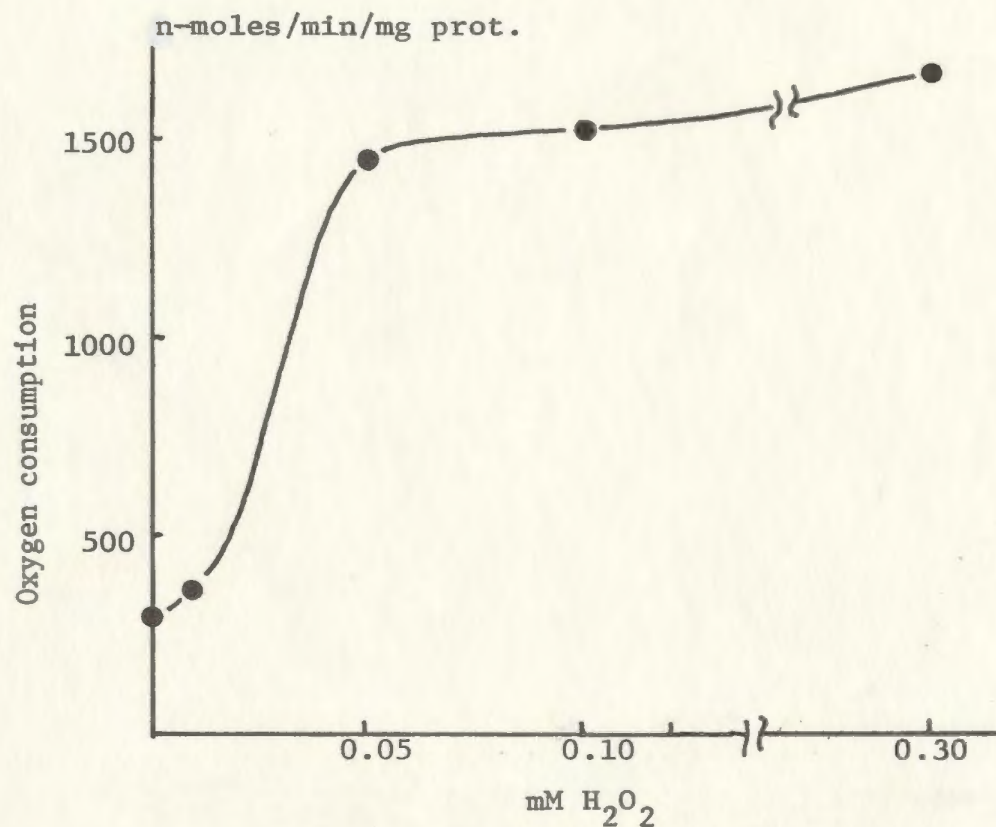
As shown in Figure 17, NADPH oxidase activated by 0.05 mM hydrogen peroxide appears to be cyanide resistant, since even 2 mM KCN does not appreciably inhibit the oxidase activity. In fact, low concentrations of KCN slightly stimulated the activity. On the contrary, only about 10% of the 2,4-DCP-stimulated NAD(P)H oxidase activity remained when 2 mM KCN was present in the reaction medium (Figure 18).

TABLE XI: Purification of myeloperoxidase (Guaiacol Peroxidase)

	<u>μ-moles/min/mg protein</u>
(a) Broken cells	1.39
(b) Granules	4.00
(c) Supernatant	5.68
(d) Peak fractions from column	40.4

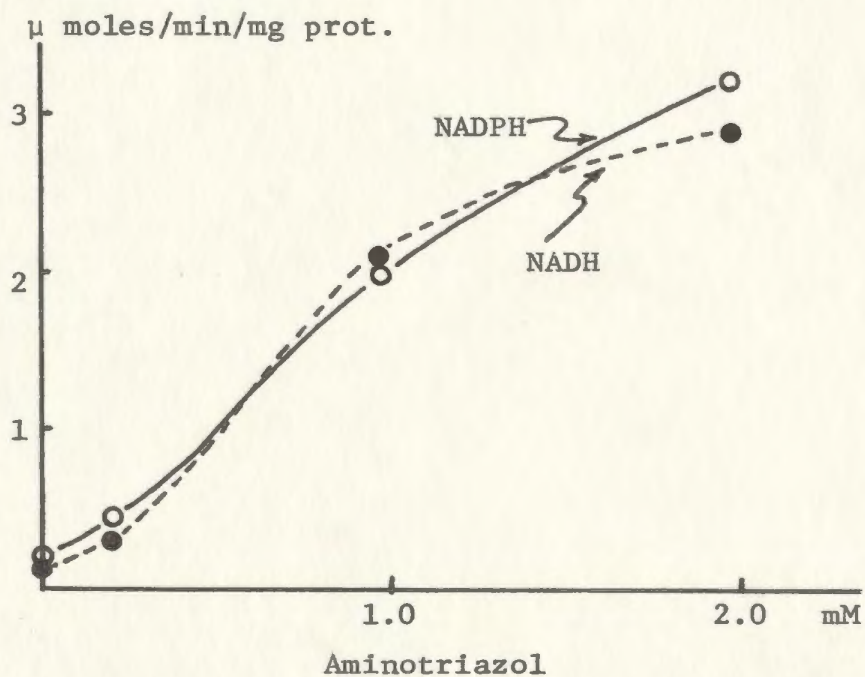
- (a) Broken with CETABRON (final concentration 0.05%)
- (b) After centrifugation and rupture with CETABRON
(final concentration 0.05%)
- (c) After removal of CETABRON-insoluble protein of
granules

FIGURE 15: Effect of hydrogen peroxide on myeloperoxidase (NADPH oxidase)



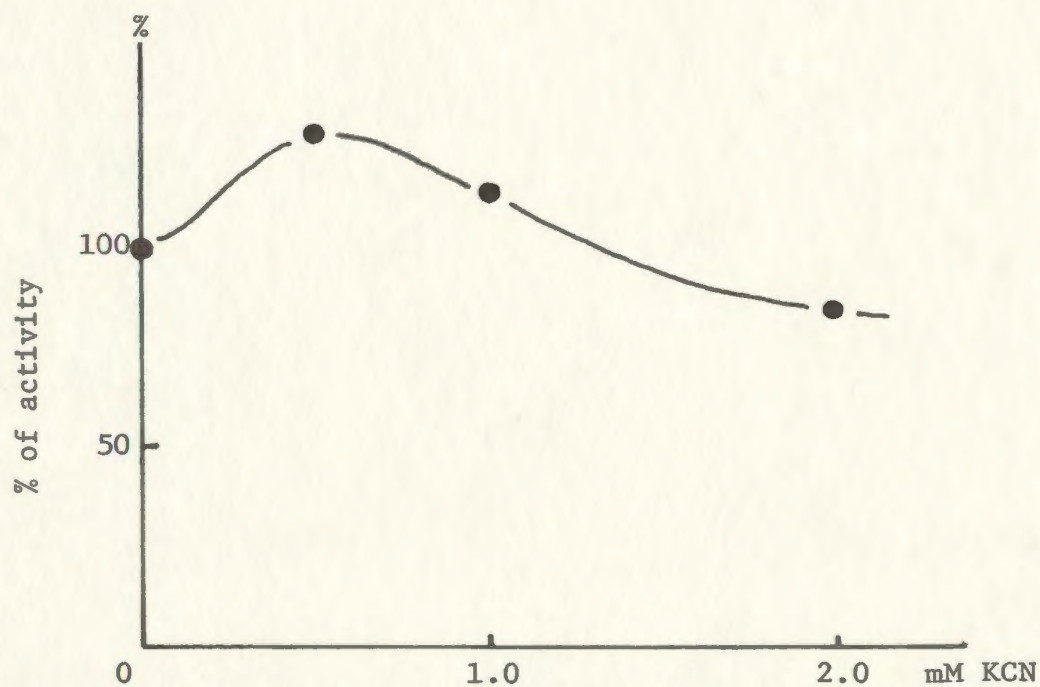
The NADPH oxidase activity was assayed at 37°C using an oxygen electrode. The medium contained 0.015 mg protein of purified myeloperoxidase, 0.5 mM $MnCl_2$, 1 mM NADPH, 50 mM phosphate buffer (pH 6.0) and various amounts of H_2O_2 . The initial rate of oxygen uptake was measured.

FIGURE 16: Effect of Aminotriazol on NAD(P)H oxidase activity of partially purified peroxidase



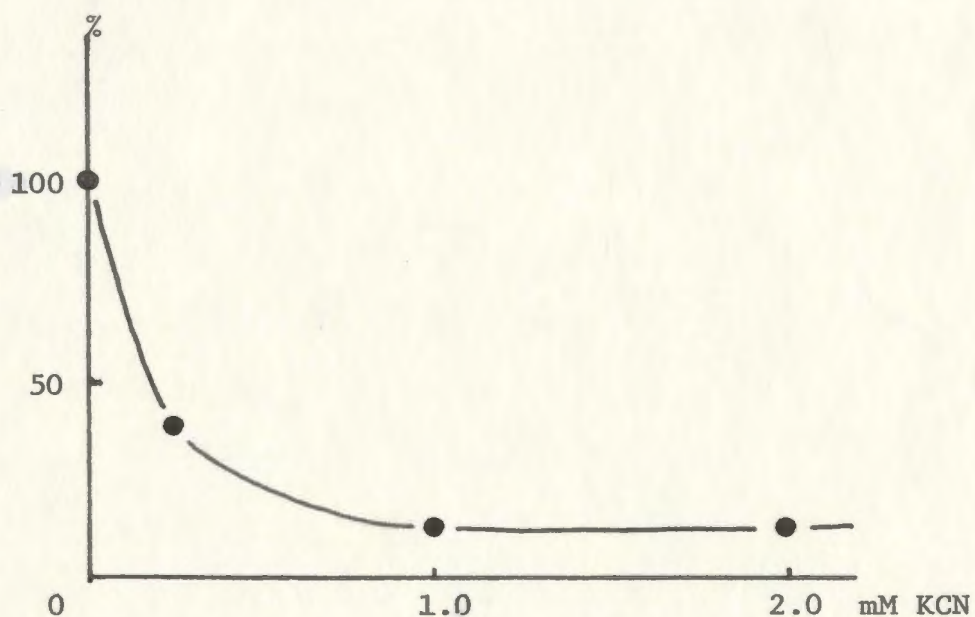
The assay conditions were identical to those in Figure 15 except various amounts of aminotriazol were added instead of hydrogen peroxide.

FIGURE 17: Effects of cyanide on NADPH oxidase activity of intact leukocytes



The assay conditions were identical to those in Figure 15 except the concentration of H_2O_2 was 0.05 mM and various amounts of KCN were added.

FIGURE 18: Effect of cyanide on the 2,4-DCP-stimulated NADPH oxidase activity of partially purified myeloperoxidase



The 2,4-DCP-stimulated NADPH oxidase activity was measured spectrophotometrically in the reaction mixture containing 0.015 mg protein of partially purified myeloperoxidase, 0.5 mM MnCl_2 , 1 mM 2,4-DCP, 0.1 mM NADPH, 50 mM phosphate buffer (pH 6.0) and various amounts of KCN at 37°C in final volume 1.5 ml. 100% of rate was 2.8 $\mu\text{moles/min/mg}$ protein.

5. The reduction of NBT during the oxidase reaction

When NBT (Nitroblue tetrazolium) was added to the NADH oxidase assay medium for HRP, the NBT was rapidly reduced resulting in the formation of a blue product (Table XII). The rate of NBT reduction was proportional to the amount of HRP added. A short lag period was observed after the reaction was initiated by 2,4-DCP, so that the maximum rate was measured as the reaction rate. The results in Table XII clearly show the dependence of the NBT reduction on the presence of manganese and 2,4-DCP. However, the reduction of NBT by peroxidase was strongly inhibited by cyanide. The NADPH also reduced NBT as effectively as NADH. In the case of leukocytes, disrupted with Triton X-100, there was a much more rapid reaction, indicating that NBT was also reduced by granule myeloperoxidase.

TABLE XII: Reduction of NBT

<u>Horseradish Peroxidase</u> ^{a)}	
<u>Conditions</u> ^{b)}	<u>O.D. Change at 560 nm</u> ^{c)} (OD/min/10 μ g peroxidase)
NADH, NBT, $MnCl_2$, 2,4-DCP	0.22
NADH, NBT, $MnCl_2$	0.01
NADH, NBT, 2,4-DCP	0.01
NADH, NBT, $MnCl_2$, 2,4-DCP, KCN(0.2 mM)	0.03
NADH, NBT, $MnCl_2$, 2,4-DCP, KCN(1.0 mM)	0.02
NADPH, NBT, $MnCl_2$, 2,4-DCP	0.23

Leukocytes

<u>Conditions</u>	<u>O.D. Change at 560 nm</u> (OD/min/100 μ g protein)
NADH, NBT, $MnCl_2$, 2,4-DCP	0.04
Triton X-100 (0.1%), NADH, NBT, $MnCl_2$, 2,4-DCP	0.64

a) Reaction mixtures were identical to those described in Figure 13(b) except for the further addition of 50 μ M NBT.

b) Reactions were initiated by the addition of 2,4-DCP.

c) Rate of reactions were obtained using the linear portion of absorbance increase at 560 nm.

DISCUSSION

In a previous chapter it was demonstrated that NAD(P)H oxidase was strongly activated by 2,4-DCP and MnCl_2 , suggesting that the formation of hydrogen peroxide by leukocytes is associated with myeloperoxidase. A role for myeloperoxidase in the hydrogen peroxide generating system of leukocytes has been thought unlikely since cyanide inhibits peroxidase activity. However, in the presence of cyanide, myeloperoxidase exhibits an oxidase reaction. The effect of cyanide on the peroxidase reaction has been investigated thoroughly by B. Chance in terms of the kinetics of the enzyme-substrate compound (62), but the presence of an oxidase activity was not then realized.

As shown in Figure 13, the extent of the inhibition of HRP by cyanide depends on the reaction system. Thus, the NADH oxidation reaction in the absence of 2,4-DCP is essentially cyanide resistant, but it becomes cyanide sensitive in the presence of 2,4-DCP. This is in agreement with the observation of Akazawa and Conn who reported that 0.17 mM cyanide inhibits approximately 50% of the NADH oxidase reaction of HRP activated by resocinol (46).

Furthermore, the evidence presented in this chapter can explain well the cyanide insensitive NADPH oxidizing activity reported by Rossi's group. That is, it seems reasonable that the leukocytic NAD(P)H oxidase activity is assoc-

iated with myeloperoxidase since it is well established that the reaction mechanism of myeloperoxidase is more or less the same as that of the HRP in terms of the NAD(P)H oxidase reaction (further details of the reaction mechanism will be discussed later).

As described in chapter IV, intact leukocytes had a 2,4-DCP-activated NAD(P)H oxidase which is further stimulated by manganese indicating that the peroxidase is associated with an intraleukocytic hydrogen peroxide generation system. However, guaiacol peroxidase activity was very low in intact leukocytes and high activities were found only when the cells were disrupted. In order to measure the peroxidase activity using the same oxidase activators and hydrogen donor, NADH:H₂O₂ peroxidase was measured under anaerobic conditions so as to eliminate the effects of the NADH oxidase activity. Hogg and Jago have demonstrated that peroxidase catalyzes the oxidation of NADH by H₂O₂ (63). When intact leukocytes were examined by this assay method, NADH peroxidase activity was very low but was increased almost twenty fold when leukocytes were disrupted. On the contrary, the NADH oxidase activity was increased only three fold by disruption.

Although it seems difficult to explain the lack of peroxidase activity in intact leukocytes, there are three possibilities:

(i) Plasma membrane-located oxidase has a high NAD(P)H oxidizing activity but a small peroxidase activity.

(ii) The NAD(P)H oxidase activity of intact leukocytes is not due to myeloperoxidase

(iii) Since the NAD(P)H oxidation reaction is a type of free radical chain reaction, NAD(P)H may not necessarily form a complex with the enzyme.

In the next chapter the intracellular distribution of NAD(P)H oxidase activity is studied to clarify these speculations. In any case, studies presented here clearly show that myeloperoxidase can act as a cyanide-resistant NAD(P)H oxidase in leukocytes.

An additional finding was the ability of myeloperoxidase to catalyze the NBT reduction reaction which is a popular cytochemical method used clinically to detect CGD (20) and other leukocyte defects (64, 65). It has been thought that NADH oxidase or diaphorases are responsible for the NBT reaction. Baehner and Nathan have demonstrated that normal leukocytes are capable of NBT reduction during phagocytosis, unlike CGD leukocytes which are not able to form hydrogen peroxide (20). Biochemical information on the NBT test, however, has not been established as yet since the activity is poor when the cytosol and granule fractions of leukocytes are incubated with NBT and NADH (66). However, as demonstrated in Table XI, purified peroxidase or disrupted leukocytes show very powerful NBT reducing

activity when Mn^{2+} and 2,4-DCP are added to the reaction system. These experimental observations suggest that the intragranular NBT reduction of leukocytes is associated with myeloperoxidase.

CHAPTER VI: THE INTRACELLULAR LOCATION OF NAD(P)H OXIDASE IN THE LEUKOCYTES

In previous chapters it was suggested that an oxidase activity associated with the plasma membrane and an intragranular peroxidase are both involved in the production of H_2O_2 in leukocytes. In the present chapter, experiments are described in which the subcellular distribution of 2,4-DCP-stimulated NAD(P)H oxidase (indicated as NAD(P)H oxidase in this chapter) is determined by a density gradient subfractionation technique.

Fractionation of leukocytic homogenate by differential centrifugation and by continuous density gradient centrifugation

For continuous density gradient centrifugation, leukocytes were collected as described in chapter I; the cells were washed with 0.25M sucrose solution containing 5 mM Na_2CO_3 (pH 8), resuspended in 0.35M sucrose containing 5 mM Na_2CO_3 and 2000 units of heparin, and the suspension was homogenized with a Potter type homogenizer for 3 to 4 minutes until it became clear. To remove unbroken cells and nuclei, the solution was centrifuged at $1,500 \times g$ for 10 minutes. The supernatant was further centrifuged at $105,000 \times g$ for 60 minutes to obtain granules and membranes. The granules plus membranes sediment was resuspended in 0.25 M sucrose (5 ml) and layered on 30 ml of a continuous sucrose

gradient (10% to 60% sucrose). The ultracentrifugation was carried out for 14 hours at 25,000 r.p.m. in the SW-27 rotor. Fractions (2 ml) were collected from the top of the centrifuge tube.

Enzyme assays

Lactate dehydrogenase assays were carried out by Kornberg's method (51). 5'-Nucleotidase (67), guaiacol peroxidase (68), alkaline phosphatase (69) and β -glucuronidase(70) were assayed according to published methods, which are commonly used for leukocyte preparations. All enzyme assays were carried out in the presence of 0.05% Triton X-100. The reaction mixtures contained 0.25 M sucrose for the peroxidase and NAD(P)H oxidase latency measurements. NADPH oxidase activity was measured in the presence of 2,4-DCP according to chapter 1.

RESULTS AND DISCUSSION

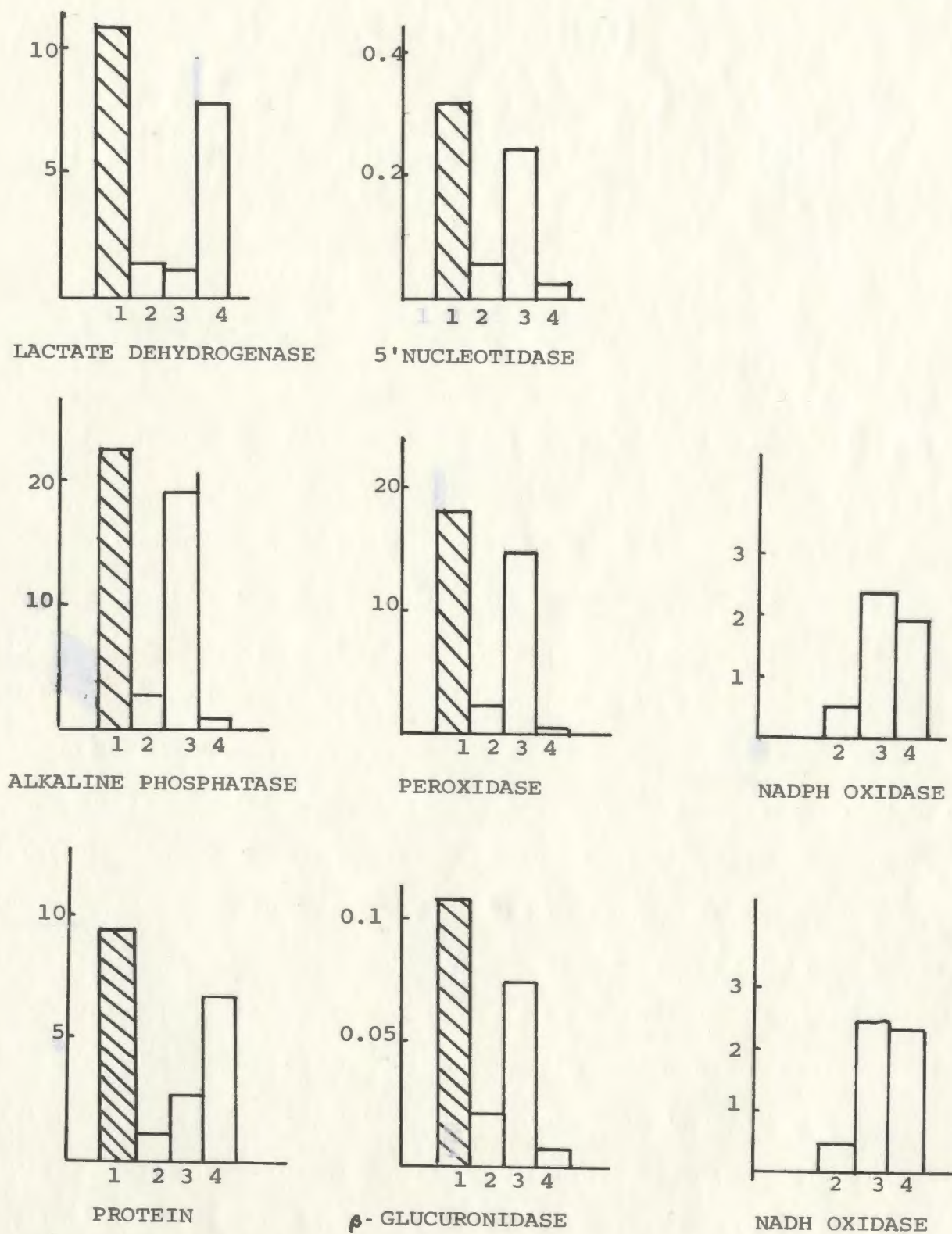
Intracellular distribution of the NAD(P)H oxidase using differential centrifugation followed by continuous sucrose gradient centrifugation of the granule-membrane fraction

Fractionation of the leukocyte homogenate by differential centrifugation followed by continuous sucrose gradient centrifugation of the granule-membrane fraction indicates the distribution of a NAD(P)H oxidase. The distribution of cytosol, plasma membrane, lysosomes, specific granules and azurophil granules using the enzyme markers lactate dehydrogenase, 5'-nucleotidase, alkaline phosphatase and guaiacol peroxidase, respectively, are shown in Figures 19 and 20. Lactate dehydrogenase was mainly found in the cytosol fraction. Most of the granule and membrane marker enzymes were sedimented after centrifugation at 105,000 x g for 60 minutes (Figure 19). It was interesting that the NADH and NADPH oxidase appeared in both the cytosol fraction and in the granule-membrane fraction. The NAD(P)H oxidase in the cytosol fraction showed a long lag period (5 to 10 minutes) before maximal activity was reached. In such cases the maximum rates were used as a measure of enzyme activity. The recoveries of all enzyme activities were greater than 90%, although the total activity of NAD(P)H oxidase was difficult to ascertain because of the unusual lag period observed for the oxidase activity of the homo-

genates.

The continuous sucrose density gradient (Figure 20) indicates that there is an NAD(P)H oxidase activity associated with the plasma membrane, although the peroxidase containing fractions possess most of the NAD(P)H oxidase activity. Furthermore, the peroxidase and oxidase activity of the peroxidase containing fractions were enhanced by Triton X-100 and showed a similar latency, whereas the NAD(P)H oxidase activity of the peroxidase-free cytosol fraction was not activated by Triton X-100. There are two possible explanations for the cytosol activity. Firstly, some of the cytoplasmic oxidase may be displaced from the plasma membrane and represent an artifact of homogenization and fractionation procedures. Secondly, there may be two separate NAD(P)H oxidases, one located in the plasma membrane and one located in the cytosol. The lag period was probably due to the presence of either catalase, superoxy dismutase or glutathione in the cytosol fraction which prevented the accumulation of H_2O_2 required to initiate the NADH oxidase.

Figure 19



1. Total activity
2. Unbroken cells and nuclei.
3. Granule fraction
4. Cytosol fraction

Protein content is expressed in mg derived from 10^8 leukocytes. All enzyme activities are: μ -moles/ min/ 10^8 leukocytes.

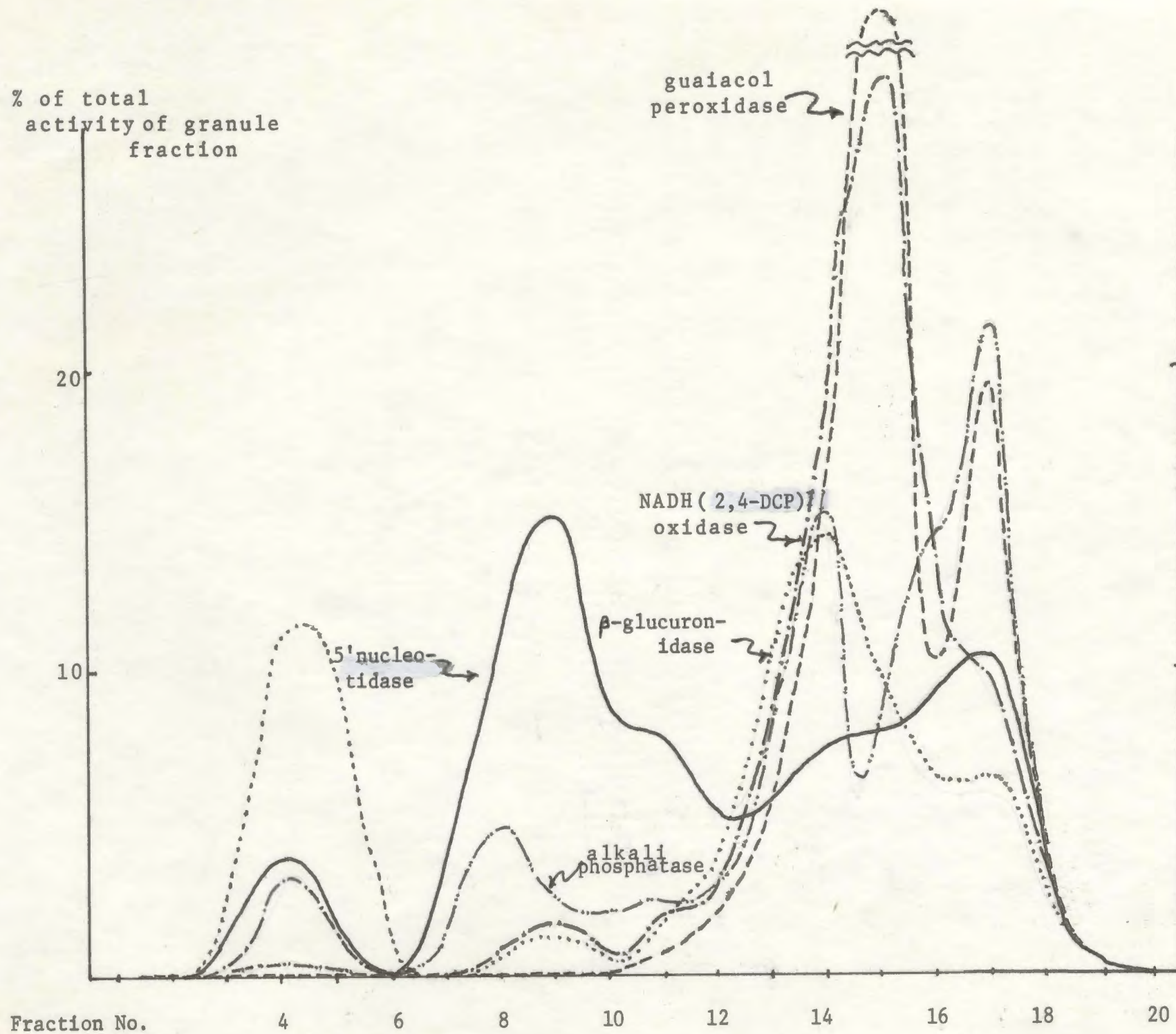


Figure 20

CONCLUSION

Although the importance of leukocytes in the host defence system against invading microorganisms has been pointed out for over a century, biochemical information on its mechanism has not yet been clarified. At present the microbicidal activity is believed to be due to the action of hydrogen peroxide with myeloperoxidase and electron donors, such as chloride, iodide or thiocyanate ions. Particle engulfment causes a large increase in oxygen uptake resulting in hydrogen peroxide formation and an enhanced rate of glucose oxidation by way of the HMP pathway. A release of peroxidase from the leukocytic granules into the phagocytic vacuoles as well as the increased formation of hydrogen peroxide could explain the microbicidal activity of phagocytizing leukocytes. However, conflicting reports have been published as to the intracellular site and mechanism of the formation of hydrogen peroxide since both NADH oxidase and NADPH oxidase systems have been invoked. Furthermore, although it is known that leukocytes in chronic granulomatous disease (CGD) are defective because of an inability to form hydrogen peroxide following phagocytosis, the actual enzymatic lesion is still not known in spite of extensive investigation.

Studies on intact and subfractioned leukocytes have led to the discovery of a new hydrogen peroxide-prod-

using oxidase which may be located in the plasma membrane of leukocytes and the key enzyme of the leukocytic hydrogen peroxide generating system. The properties of the oxidase are as follows:

(a) Both NADH ($K_m = 1.2$) and NADPH ($K_m = 0.5\text{mM}$) can serve as hydrogen donors for hydrogen peroxide formation.

(b) The oxidase is activated when leukocytes are swollen in a hypotonic medium even though the cells have not been disrupted. This activation is readily reversible.

(c) Addition of 2,4-dichlorophenol (2,4-DCP) to an isotonic leukocyte suspension stimulates both oxygen uptake and NAD(P)H oxidase activity. The intactness of the cells is not affected by the 2,4-DCP.

(d) Oxygen consumption by leukocytes in the presence of 2,4-DCP, NAD(P)H, Mn^{2+} , and KCN results in hydrogen peroxide formation.

(e) NAD(P)H oxidizing activity, enhanced by 2,4-DCP or hypotonic treatment, is inhibited by p-CMB which does not permeate the plasma membrane.

(f) The addition of digitonin (at concentrations which do not affect the permeability of the plasma membrane to NADH or trypan blue) to leukocytes results in a burst of oxygen consumption.

(g) Externally added oxidized cytochrome c is efficiently reduced during the burst of oxygen consumption. This reduction is blocked by superoxide dismutase.

(h) The NAD(P)H oxidase system associated with the

plasma membrane fraction of the leukocytes has no peroxidase activity. On the other hand, pure horseradish peroxidase and partially purified myeloperoxidase has both oxidase and peroxidase properties.

Localization of NAD(P)H Oxidizing Activity

One of the unanswered questions is the location of the hydrogen peroxide formation sites in leukocytes. The data published on the NADH and NADPH oxidase system are conflicting, since these activities have been demonstrated both in a granule fraction and in a cytosol fraction by different workers using different experimental conditions.

Rossi's group has maintained that the NADPH oxidase is distributed in intraleukocytic granules and is activated during phagocytosis. On the other hand, Karnovsky's group have suggested that cytosol located NADH oxidase plays a central role in hydrogen peroxide production. Furthermore both groups have a different explanation for the origin of hydrogen peroxide in the phagocytic vacuoles. In addition, the studies of both groups were carried out with broken leukocytes. Since leukocytes are not disrupted by a glass-homogenizer, a commonly employed technique is the use of ion-free sucrose which disrupts the plasma membrane, possibly due to the removal of divalent cations. However, the effect of this treatment on the plasma membrane-associated enzymes is not known. Therefore, studies

using intact leukocytes may avoid these problems.

The experiments reported here were carried out with intact leukocytes either under hypotonic conditions or after digitonin treatment. Furthermore, as shown in chapter IV, the hydrogen peroxide forming enzyme was investigated using 2,4-DCP with intact leukocytes in isotonic suspension. Finally, results obtained with intact leukocytes were compared with purified enzymes and subcellular particles.

The experimental results indicating the site of H_2O_2 formation in the previous chapter are summarized as follows:

(a) A hypotonic medium greatly enhances the production of H_2O_2 by intact leukocytes during the oxidation of added NAD(P)H without appreciable disruption of the cells.

(b) Hydrogen peroxide formation from NAD(P)H oxidation by intact leukocytes is strongly influenced by p-CMB which does not penetrate the plasma membrane.

(c) Although low concentrations of digitonin do not disrupt the cell membrane, leukocytic respiration is stimulated greatly. Furthermore the digitonin-treated leukocytes reduce cytochrome c although the cytochrome c can not penetrate the leukocyte plasma membrane. The exclusion of trypan blue by the digitonin-treated leukocytes was a further observation indicating that the cell membrane was still intact. At higher concentrations of digitonin the cells became leaky and this was accompanied by an inactivation of

respiration.

(d) Intact leukocytes under isotonic conditions generate hydrogen peroxide in the presence of NAD(P)H, Mn^{2+} , 2,4-DCP and KCN.

(e) The oxidase activity is distributed in cytosol, plasma membrane and granule fractions. Although granule located myeloperoxidase has NAD(P)H oxidase activity, there is a 2,4-DCP stimulated NAD(P)H oxidase associated with the plasma membrane fraction which has no peroxidase activity.

From these experimental observations, it seems reasonable to suggest that the site of hydrogen peroxide formation is associated with the plasma membrane of leukocytes and that the enzyme is activated during phagocytosis due to morphological alterations of the plasma membrane. Alternatively, one can also speculate that the granule located myeloperoxidase can also generate hydrogen peroxide. However, we cannot explain why the respiratory burst is initiated before the myeloperoxidase - granules fuse with phagosomes, unless some myeloperoxidase is distributed on the plasma membrane.

Proposed Sequence of Events leading to H_2O_2 Production during Phagocytosis

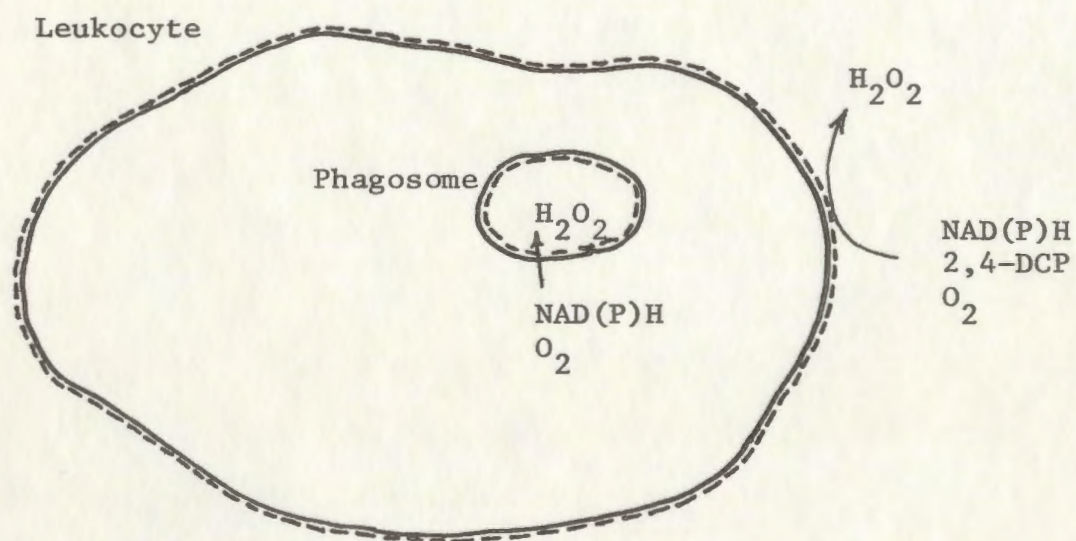
It seems likely that the hydrogen peroxide generating system is distributed on the outside surface of the plasma membrane of leukocytes, and that the enzyme is act-

ivated during the phagocytizing process due to morphological alterations of the plasma membrane. Furthermore, the hydrogen peroxide producing enzymes are transferred from the plasma membrane to the inside of the phagocytic vacuole, as shown in Figure 21. This assumption could explain the controversy between Karnovsky and Rossi. That is, since the hydrogen peroxide producing enzymes are associated with the plasma membrane as well as with peroxidase-rich particles, methods to break the cell membrane might affect the distribution of the enzymes. In any case, an enzyme which has a high oxidase activity and is associated with the plasma membrane plays a central role in hydrogen peroxide production during the phagocytic process.

The location of the H_2O_2 generating system as an ecto-enzyme of the plasma membrane would mean that, after invagination as a result of phagocytosis, the system would become located on the inside surface of the phagosome. The activated H_2O_2 generating system could now generate high levels of H_2O_2 inside the vacuole in the vicinity of the bacteria because of the absence of catalase (59) and glutathione peroxidase. H_2O_2 diffusing out of the vacuole into the cell would be readily decomposed by intracellular catalase or glutathione peroxidase.

The oxidase would normally use intracellular NADPH or NADH of the cytosol and although the plasma membrane is impermeable to NAD(P)H, the phagocytic vesicle may

FIGURE 21.



be permeable to NAD(P)H as phagocytosis is accompanied by lipid turnover (72). However the respiratory burst of leukocytes in hypotonic medium or on addition of membrane active agents (eg. digitonin), indicates that the oxidase is probably located on both surfaces or traverses the membrane so that the oxidase could react with intracellular NAD(P)H or NAD(P)H added externally.

Although the distribution and some properties of the hydrogen peroxide producing system are now clear, this work is only the first step in the explanation of the intraleukocytic microbicidal action and many further problems remain to be investigated. First of all, since 2,4-DCP is an unphysiological compound, the trigger mechanism for the oxygen burst during phagocytosis still needs to be identified. This mechanism could involve superoxide radical formation by a flavoprotein or iron-sulfur-protein, which may be sensitive to p-CMB. The possibility also arises that a p-CMB sensitive NAD(P)H dehydrogenase is involved which reduces the peroxidase and thereby stimulates the oxidase activity (73). Secondly, the enzyme defect of CGD leukocytes needs to be investigated. In CGD leukocytes it has been reported that the total peroxidase level is normal (74). The possible causes of this disease might include the following five possibilities:

(a) The plasma membrane located oxidase is deficient.

(b) The trigger mechanism producing the superoxide-radical is absent.

(c) The hydrogen donor or phenolic activator is missing.

(d) The dehydrogenase that activates the oxidase is missing.

(e) Inhibitors of the oxidase are present.

The plasma membrane located oxidase raises interesting enzymological aspects. Myeloperoxidase has been demonstrated to have two subunits (75) and several isoenzymes of the myeloperoxidase exist in the leukocyte (60, 76). As described in chapter VI, the plasma membrane-associated oxidase has high oxidase activity and very low peroxidase activity.

Other peroxidases with a 2,4-DCP activated NAD(P)H oxidase activity include lactoperoxidase (77), uterine peroxidase (78, 79) and thyroid peroxidase (80). The physiological oxidase activator and peroxidase hydrogen donor could be oestradiol (81) for uterine peroxidase and tyrosine (82) for thyroid peroxidase. It is believed that the uterine peroxidase is involved in oestradiol metabolism whilst the thyroid peroxidase is involved in thyroxine biosynthesis.

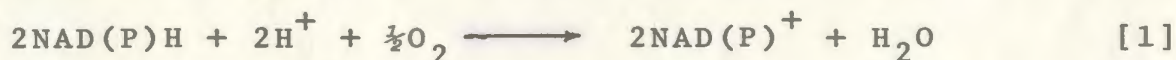
Reaction Mechanism of Peroxidase

The results presented show that guinea pig leukocytes produce H_2O_2 as the principal product of oxidation of NADPH or NADH with 2,4-dichlorophenol in the presence of 2 mM KCN. A stoichiometry of one mole H_2O_2 per mole of NAD(P)H was observed. There are several similarities between the oxidase activity of intact leukocytes and the oxidase activity of peroxidase. The oxidation of pyridine nucleotides by peroxidases shows similar rates for NADH and NADPH and is enhanced by catalytic amounts of Mn^{2+} and phenols (46); is enhanced by H_2O_2 (80); is inhibited by superoxide dismutase and catalase (57) and is inhibited by some redogenic substrates (83). The role of the myeloperoxidase in the hydrogen peroxide forming mechanism of the leukocyte was suggested by Roberts and Quastel (44) but was considered unlikely by other investigators because the leukocyte mechanism is insensitive to cyanide and the NADPH oxidase activity of leukocyte granules is enhanced by aminotriazole, whereas the myeloperoxidase is readily inactivated by cyanide and aminotriazole (24). However the oxidase and peroxidase activities of myeloperoxidase are affected differently by cyanide and aminotriazole (see chapter 5)(71).

The oxidation reaction of horseradish peroxidase was first pointed out by Akazawa and Conn (46). Since then several workers, especially Yamazaki's group, have invest-

igated the physiochemical mechanism (83-92). Traditionally, the physiological role of myeloperoxidase has been understood as a hydrogen peroxide decomposing enzyme. However, the remarkable fact is that myeloperoxidase is also able to produce hydrogen peroxide from molecular oxygen. In contrast, the oxidase properties of liver microsomal cytochrome P450 were established first, and the peroxidase properties of cytochrome P450 were demonstrated only recently by Hrycay and O'Brien (93, 94).

According to Yamazaki, both myeloperoxidase and horseradish peroxidase form compound III during NAD(P)H oxidation. Compound III is thought to be a form of the peroxidase which contains "activated oxygen" and oxidation state of peroxidase is +6. Overall, the reaction could be visualized as follows:



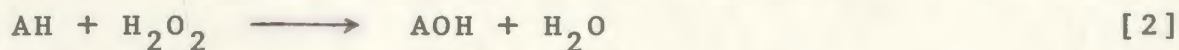
which is made up of an oxidase component-



and a peroxidase component-



The details are described later. This oxidation reaction is stimulated by manganese, and some phenols further increase the activity. The oxidase reaction is a type of chain reaction different from the well known hydroxylation reactions of peroxidase:



Quastel et al. have already suggested the possibility that the NAD(P)H oxidation reaction [1] is carried out by intraleukocytic myeloperoxidase, since Mn^{2+} stimulates the oxidation of NAD(P)H by a dialysed homogenate of leukocytes (44,45). Recently Patriarca et al. found that NADPH oxidase and myeloperoxidase activities were similarly distributed on a density gradient (24). Nevertheless, they concluded that the NADPH oxidation activity was not derived from myeloperoxidase for the following reasons:

(a) NADPH oxidation in phagocytizing leukocytes is cyanide resistant whereas peroxidase (guaiacol peroxidase activity) is fully inhibited by cyanide.

(b) Although phagocytosis markedly stimulates the NADPH oxidase activity, appreciable activation of myeloperoxidase does not occur.

(c) In CGD leukocytes, peroxidase activity is normal, (95) but the burst of oxygen consumption associated with phagocytosis in leukocytes is not observed.

(d) Aminotriazole inhibits the (guaiacol) peroxidase activity in granules but stimulates the NADPH oxidase activity.

Unfortunately, the susceptibilities of the peroxidase reaction and the oxidase reaction to inhibition or activation by different reagents were not studied. As

discussed in chapter V , purified horseradish peroxidase can act as a NAD(P)H peroxidase as well as a NAD(P)H oxidase. However, their responses to activators and inhibitors indicate different reaction mechanisms. Therefore, Patriarca's results do not necessarily exclude the possibility that the two activities are catalyzed by the one enzyme.

The mechanism of hydrogen peroxide formation by horseradish peroxidase or myeloperoxidase could involve the intermediate superoxide-radical anion and ferrous peroxidase as shown in Figures 22 and 23. The detailed mechanism of the chain reaction as proposed by Yamazaki et al. is shown in Figure 22. This reaction involves the NAD free radical and superoxide radical anion in the formation of compound III. Since the reaction in Figure 23 is an oxygen consuming reaction, it does not occur under anaerobic conditions. On the other hand, the peroxidase reaction is observed anaerobically in the presence of hydrogen peroxide.

Possible mechanisms for the oxidase reaction and the peroxidase reaction of myeloperoxidase are suggested in Figure 23. The numbers in bold type refer to the oxidation state of the iron in the peroxidase.

FIGURE 22: Chain Reaction Mechanism for NADH Oxidation as Proposed by Yamazaki et al.

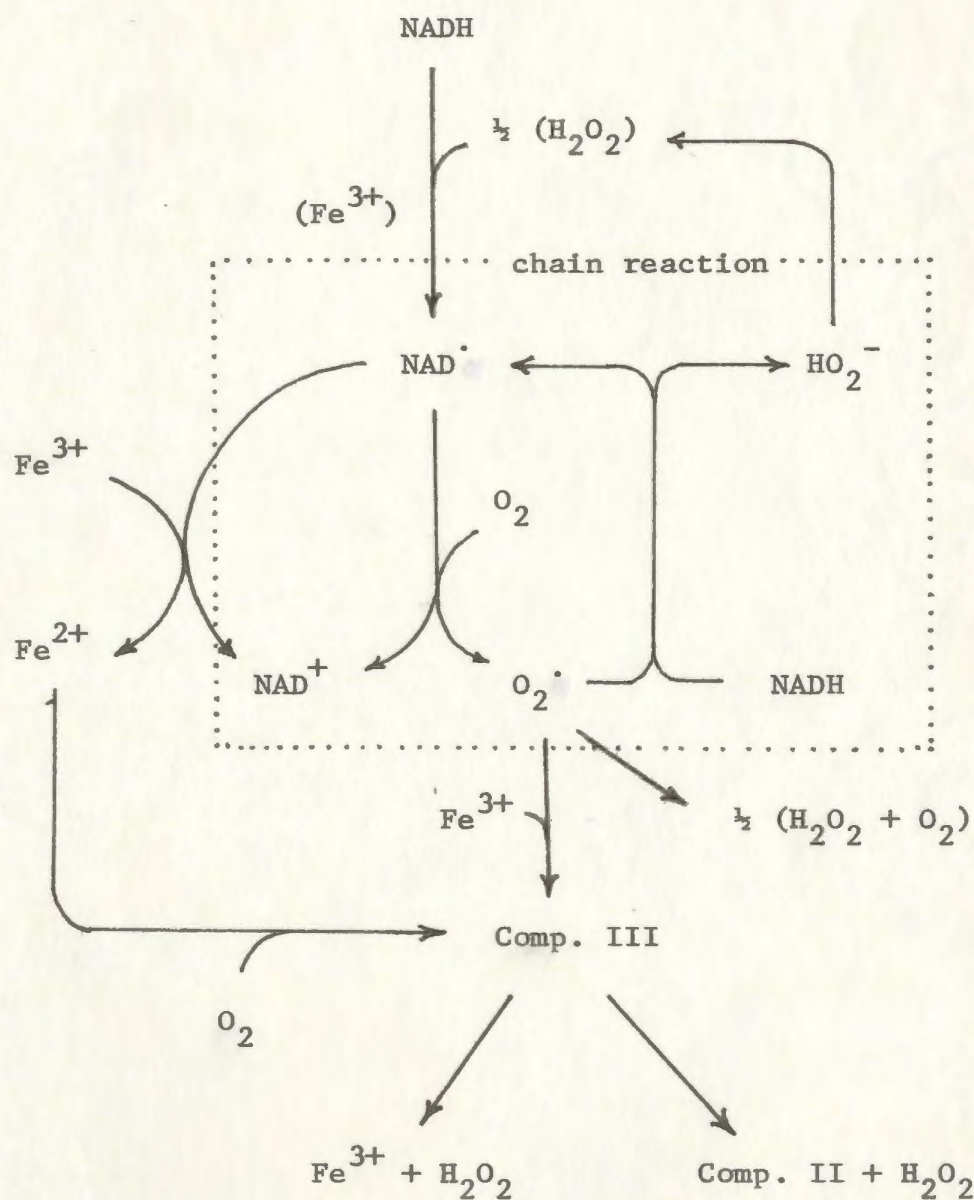
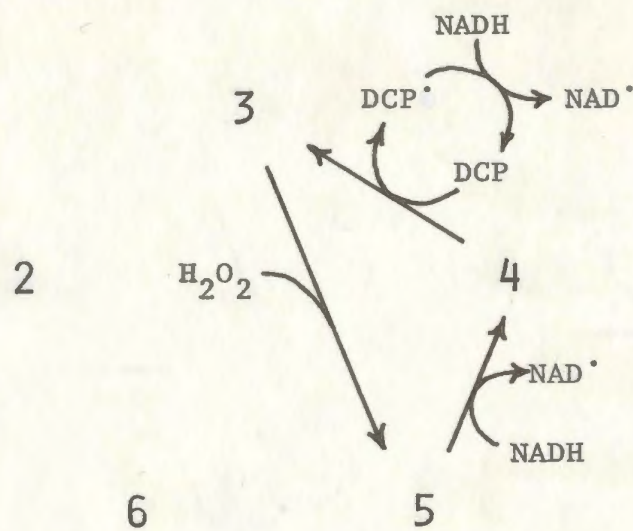
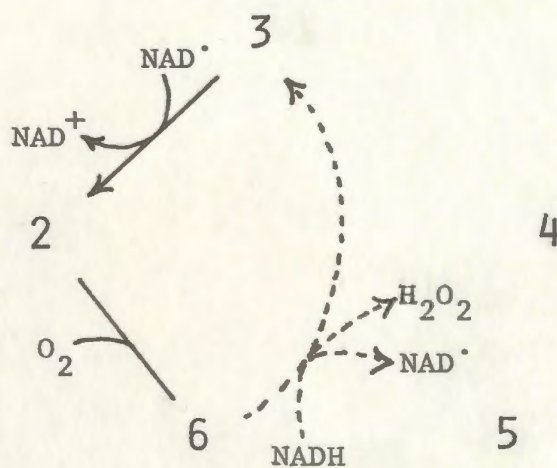


FIGURE 23. Possible Mechanism of Peroxidase and Oxidase Reaction of Peroxidase



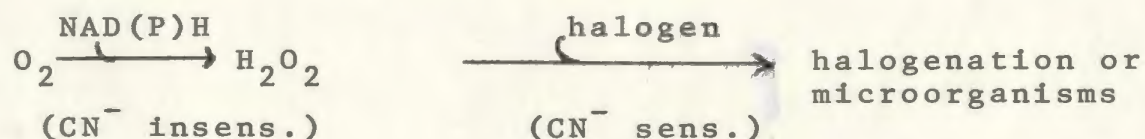
Peroxidase reaction



Oxidase reaction

The mechanism of the peroxidase reaction is well established and is believed to occur via $(3 \rightarrow 5 \rightarrow (4) \rightarrow 3)$ using different substrates (96, 97) where these numbers refer to the oxidation state of iron. In addition, the reaction $(3 \rightarrow 5)$ is effectively inhibited by cyanide. At present, it is uncertain whether the stimulation by phenols is due to the activation of $(5 \rightarrow 4)$ or $(4 \rightarrow 3)$. In any case, the peroxidation reaction proceeds via $(3 \rightarrow 5 \rightarrow (4) \rightarrow 3)$ and phenols activate $(5 \rightarrow (4) \rightarrow 3)$ forming a phenoxy radical (61).

Klebanoff's work raises the interesting point that the microbicidal action of myeloperoxidase in the presence of hydrogen peroxide and halogen compounds would be the cyanide sensitive peroxidase part of the following reaction:



The role of myeloperoxidase in the cyanide sensitivity of bactericidal action is well established (98). On the other hand, the oxidation of NAD(P)H by the oxidase reaction of myeloperoxidase might be considered an important process for the formation of hydrogen peroxide for the halogenation reaction.

Evidence that myeloperoxidase activity could not account for the cyanide resistant NAD(P)H oxidase activity includes (i) intact leukocytes had a very low guaiacol peroxidase with the same latency (93%) on disruption of the cells as the cytosol located lactate and malate dehydrogenase. Clearly little or no myeloperoxidase existed as an ecto-

enzyme of the plasma membrane. Cytochemical studies indicate that myeloperoxidase is located in the azurophil granules of the leukocyte (49). (ii) The myeloperoxidase activity of the leukocytes were unaffected by a hypotonic medium although NAD(P)H oxidase was activated. (iii) Cell fractionation studies using discontinuous gradient centrifugation showed that much of the NAD(P)H oxidase activity was associated with lighter fractions than the myeloperoxidase containing granules. (iv) Myeloperoxidase deficient leukocytes show a normal metabolic response to Phagocytosis (100) and leukocytes from patients with chronic granulomatous disease show no respiratory burst but cytochemical studies indicate that myeloperoxidase release is normal (95)(99). Clearly the plasma membrane located NAD(P)H oxidase is either an isoenzyme of myeloperoxidase with unusually high oxidase and low peroxidase activities (101) or another peroxidase. According to the oxidase-peroxidase mechanism of Yamazaki et al. (91), an NAD(P)H reductase activity, in association with the oxidase and capable of catalyzing the reduction of the peroxidase or the oxyperoxidase intermediate, would greatly enhance the oxidase activity of a peroxidase. Alternatively, if the oxidase catalyzed the reaction between NADH and O_2^- , a chain reaction would be propagated, resulting in superoxy radical formation and consequently enhancing the activity. Recently this reaction has been demonstrated enzymatically (102).

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